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# THE CULTIVATION OF THE FREE-LIVING STAGES OF THE HOOKWORM, *ANCYLOSTOMA BRAZILIENSE* DE FARIA, UNDER ASEPTIC CONDITIONS

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*(Accepted for publication, 18th August, 1947.)*

The first two larval stages of the hookworm are entirely free-living. The eggs, which are passed out in the faeces, become mixed with soil and, after a time, the first stage larvae hatch out. These feed actively, increase in size and finally moult to give rise to the second stage larvae, which are morphologically very similar. These also increase rapidly in size and continue to feed until the changes preparatory to the second ecdysis take place. The mouth closes, certain changes take place in the oesophagus which loses its original rhabditiform appearance, and finally the cuticle separates to form a sheath about the larva. It is now in the third stage and its life history can only be continued by invading the appropriate host.

The method commonly used to enable this free-living development to take place in the laboratory is to incubate a mixture of infected faeces with earth or charcoal at 20–30° C. It was thought for many years that hookworm larvae fed on the organic matter in the faeces. The earth or charcoal was believed merely to hinder the decomposition of the faeces and to act as a diluent, permitting oxygen to reach all parts, so that development was not limited to the eggs on the surface. Authors spoke vaguely of the fine organic detritus of the faeces acting as food for the larvae. Looss (1911) said that "the normal food of Ankylostoma larvae consists of the finest solid constituents of the faeces, perhaps also of certain substances contained in them in solution." He did not believe that bacteria had a sufficient nutritive value for the larvae.

However, McCoy (1929) showed that *Ancylostoma caninum* could develop to the third stage when the only food provided was a suspension of living bacteria. He washed the hookworm eggs well, and after killing any bacteria present, inoculated them on nutrient agar cultures of a single species of bacterium. Many different species of bacteria were found to be suitable as food. Various control experiments confirmed his belief that living bacteria were the sole source of the food. For instance, growth to the third stage took place in a saline suspension of suitable bacteria, but not in bacteriologically sterile filtrates of such suspensions or in suspensions of heat-killed bacteria. To determine whether the non-living organic substances present in faeces could serve as food he inoculated with bacteria-free

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hookworm eggs, faeces that had been autoclaved for an hour at 15 lb. pressure. In two such experiments no third stage larvae grew, but in the third (which was carefully checked for the presence of living bacteria) a few larvae did come through to the infective stage. McCoy concludes: "Apparently there are substances aside from bacteria in the faeces which have a slight nutritive value for the larvae."

Lapage (1932–3) extended McCoy's work by cultivating a variety of Strongyloidea (from sheep and rabbits) to the infective stage in culture media containing living bacteria.

Glaser and Stoll (1938) approached the problem of the necessity of bacteria for the growth of Strongyloidea larvae from a different angle. They wanted sterile third stage larvae so as to provide a starting point for an attempt to reproduce the parasitic stages of the life history. Glaser and Coria (1933) had been successful in cultivating *Paramecium caudatum* in a complex medium consisting of liver extract, killed yeast cells and sterile fresh rabbit kidney; and since other animals, such as *Aëdes aegypti* (Trager, 1935) and house flies (Glaser, 1938) had also been found to develop in a similar medium, they tried a slightly modified variant for the cultivation of the free-living stages of *Haemonchus contortus* (Rudolphi), the stomach worm of sheep. They were successful; the larvae reached the third stage under completely bacteria-free conditions. However, the greatest number of infective larvae produced in any one culture was only about one-fourth of the number of the viable eggs inoculated, and abnormal forms were occasionally seen. The authors thought that their conditions were never quite ideal for the aseptic growth of these larvae.

The following work was undertaken in the hope of finding a fairly simple and easily reproducible medium in which the free-living stages of the hookworm would develop under aseptic conditions, so that physiological or biochemical studies could be made without the complications introduced by the presence of bacteria.

## EXPERIMENTAL.

The hookworm eggs for use in the following experiments were obtained from cats and dogs which had been infected with a strain of *Ancylostoma braziliense* obtained by culturing the eggs from teased-up worms. The experimental animals were kept in cages with concrete floors so as to avoid the possibility of infection with other species of hookworms.

The primary essential was to "sterilize" the eggs. First they had to be freed from the faeces as far as possible. The method used was based on that of McCoy and involved washing the eggs several times by centrifuging with water, floating them up by centrifuging in a salt solution of specific gravity 1·15, collecting them from the surface using a wide-mouthed pipette and again washing them in the centrifuge, this time with several lots of sterile tap water. Finally, they were concentrated at the bottom of a 15 or 25 ml. centrifuge tube ready for the addition of the sterilizing agent.

Some trouble was at first experienced in finding a suitable sterilizing agent. McCoy (1929) used 5 p.c. antiformin solution in 10 p.c. formalin while Glaser and Stoll had more success

with White's solution, which has the following composition: mercuric chloride, 0·25 gm.; sodium chloride, 6·5 gm.; hydrochloric acid, 1·25 ml.; ethyl alcohol, 250 ml.; distilled water, 750 ml.

Lapage used a number of methods of which 1·3 p.c. HCl and 5 p.c. antiformin in 10 p.c. formalin were the most reliable. However, none of these proved reliable in my hands. It early became apparent that the eggs were remarkably resistant to a variety of antiseptics and it was mainly a matter of finding one powerful enough to kill off all of the bacteria in a reasonable time, say 30 minutes to one hour.

A number of preliminary experiments were carried out with different sterilizing agents. Usually they were left for 30 minutes in contact with the eggs which were then washed four to five times by centrifuging with 10–20 ml. of sterile water. Finally, the sterility of the eggs was checked by inoculation on a blood agar slope and into a tube of Brewer's medium (for the detection of anaerobes) both being incubated at 37° C. overnight, followed by a week at 25° C. Meanwhile, some of the eggs (still in water) were incubated at 25° C. for 24 hours and the number of viable and non-viable eggs counted. The number of larvae plus eggs containing larvae past the tadpole stage (in practice almost all of these eggs contained nearly fully developed larvae) was taken as the number of viable eggs. Table 1 sets out a representative series of these experiments.

TABLE 1.

| Experiment. | Sterilizing solution. | Period of sterilization in mins. | No. of viable eggs. | No. non-viable eggs. | P.c. of viable eggs. | Result of sterility test. |
|---|---|---|---|---|---|---|
| 1 | White's | 20 | 54 | 496 | 9·8 | Growth |
| | 10 p.c. formalin plus 5 p.c. antiformin | 30 | 545 | 212 | 72·0 | Growth |
| 2 | White's | 40 | 79 | 82 | 49·1 | Growth |
| | 1·3 p.c. HCl | 40 | 103 | 108 | 48·8 | Growth |
| 3 | Undiluted Milton | 30 | 21 | 150 | 12·3 | Growth |
| | 10 p.c. formalin in 50 p.c. Milton | 30 | 38 | 100 | 27·5 | No Growth |
| | White's | 30 | 131 | 102 | 55·6 | Growth |
| | N/10 $KMnO_4$ plus equal vol. N/5 HCl | 5 / 25 | 67 | 151 | 30·7 | Growth |
| 4 | 10 p.c. formalin in 50 p.c. Milton | 35 | 90 | 87 | 50·8 | No growth |
| | 10 p.c. formalin in 10 p.c. Milton | 35 | 115 | 99 | 53·7 | No growth |
| | Modified White's* | 35 | 29 | 200 | 12·7 | No growth |
| | 0·05 p.c. merthiolate | 35 | No count done | | | Growth |
| | No treatment | | 126 | 86 | 59·4 | |

* $HgCl_2$ 0·1gm., HCl 2·0 ml., Alcohol 50·0 ml. distilled water to 100 ml.

The sterilizing solution consisting of 10 p.c. formalin in 10 p.c. "Milton" proved to be very reliable. "Milton" is a proprietary antiseptic which is claimed to contain 1 p.c. sodium hypochlorite and so is rather similar to antiformin (a mixture of equal parts of 15 p.c. NaOH and liquor sodae chlorinatae) though it is not so alkaline and has less available chlorine. In some of the later work eggs of Ascaridae as well as hookworm eggs were present in the material to be sterilized. In this event it was found better to add 0·5 ml. antiformin to the egg suspension followed one minute later by 10-20 ml. of the 10 p.c. formalin in 10 p.c. "Milton" solution, action for 30 minutes being allowed. The alkali in the antiformin stripped the rough albuminous outer coat from the eggs and this may have enabled the antiseptic to reach any bacteria that had been protected by this coat. In any case it proved the most reliable method.

The proportion of viable eggs obtained varied markedly from batch to batch even though the same sterilizing solution was being used for the same time. The best result was 97 p.c. viable, the worst 34 p.c. This variation probably depended on a number of factors such as the age of the faeces, the number of centrifugings, the length of time the eggs were left in the salt solution and the proportion of fertile eggs. Consequently the results recorded for different experiments in Table 1 are not directly comparable, though those within a single experiment are.

For the cultivation experiments the eggs were sterilized and washed as above. Some were inoculated on a blood agar slope and into a tube of Brewer's medium and were incubated at 37° C. overnight to see if they were sterile. A measured fraction of the egg suspension was also incubated overnight but in this case at 25° C. Next day the number of viable eggs was counted and from this the number of viable eggs in the original suspension calculated. This suspension had, meanwhile, been left at 15° C. and if the sterility tests were negative the media were then inoculated. Incubation of the sterility tests was continued at 25° C. for a minimum of seven days before they were finally regarded as negative.

The first attempts to produce a medium for the aseptic cultivation of hookworm larvae were based on McCoy's work. Although he had failed to get the larvae to develop on heat-killed bacteria, he had found a few third-stage larvae developing on autoclaved faeces in one experiment. It seemed possible that a successful medium might be made by adding suspensions of bacteria killed in various ways to sterile meat or vegetable extracts.

It was found as expected that *Ancylostoma braziliense* could develop to the third stage on media containing living bacteria. The method used was similar to that of Lapage (1932–3). The medium was Lemco broth (Fildes *et al.*, 1931) diluted with 10 volumes of distilled water and distributed in Erlenmeyer flasks to a depth of not more than a few millimetres. It was inoculated with *Bact. coli* just before the eggs were added. Practically all the viable eggs developed to the infective larval stage in this medium.

Next an attempt was made to find a suitable medium containing dead bacteria. The bacteria (*Bact. coli*) were killed in three different ways; by heating at 65° C. for two hours; by irradiation with ultra-violet light; by treatment with acetone followed by aeration of the suspension for 15 minutes at 57–60° C. to get rid of the acetone as far as possible. They were then suspended in the diluted Lemco broth mentioned above. None of the larvae reached the third stage in any of these media though in the one containing bacteria killed by ultra-violet light some developed as far as the second stage.

Since it was possible that the larvae had been utilizing some diffusible product of bacterial metabolism in the cultures containing living bacteria, further cultures were made by mixing a suspension of heat-killed bacteria with various dilutions of a Seitz filtrate of a 24- or 48-hour-culture of *Bact. coli* in broth. Controls without the *Bact coli* suspension were also put up. However, the larvae did not develop in any of these media.

Finally, since baker's yeast had been found of benefit or necessary in the sterile cultivation of a number of animals, the effect of the addition of yeast and yeast extracts was tried.

However, it was found that a suspension of baker's yeast in the dilute Lemco broth, even though the yeast cells were alive and multiplying, did not allow the larvae to develop.

McCoy had found that a wild yeast, *Torula rosea*, would not support the growth of *Ancylostoma caninum*, and suggested that the young larvae could not ingest it on account of the large size of the cells. Accordingly the next medium tried was live baker's yeast ground aseptically to colloidal dimensions and suspended in the diluted Lemco broth; but again there was no sign of development of any larvae. At the same time a medium of ground heat-killed yeast (Glaser and Stoll, 1938) plus heat-killed *Bact. coli* suspended in the dilute Lemco broth was tried but it was a failure also. Finally, two different yeast extracts were made, one as used by Gladstone and Fildes (1940) for the culture of bacteria, and the other by grinding live yeast aseptically in a little Ringer's solution, incubating overnight at 37° C. and then filtering through a Seitz filter. They were then diluted with Lemco broth (the final concentration of the Lemco broth was 1/10) and a heat-killed suspension of *Bact coli.* added. Once again no development of the larvae took place.

This approach was then abandoned. As has been mentioned, Glaser and Stoll had been successful in cultivating *Haemonchus contortus* under aseptic conditions on a semi-stiff agar medium containing ground heat-killed yeast, liver extract and fresh rabbit kidney, so it was decided to try whether the same medium could be used successfully for *A. braziliense*. The medium was made up following Glaser and Stoll's directions exactly. It was dispensed in 5 inch × ½ inch test tubes to a depth of about 1 cm., and, after inoculation, incubated at 25° C. for a minimum of 10 days, the atmosphere being kept moist so as to hinder evaporation. The number of viable eggs used in the inoculum varied from 200 to 6,000 but was usually about 500. The sterility of any positive culture was checked by making smears as well as by the inoculation of Brewer's medium and a blood agar slope.

*A. braziliense* was found to develop to the third stage on this medium. Further experiments showed that the yeast and the liver extract could be omitted without stopping the larvae from reaching the third stage; mature third-stage larvae were produced on 0·2 p.c. water agar plus sterile rabbit kidney. It is clear that the essential ingredient in Glaser and Stoll's medium is the fresh sterile rabbit kidney and this, unfortunately, is the most complex one.

The results obtained on these media were variable and always rather poor. Third stage larvae did not develop in every tube inoculated and if 5 p.c. of the viable eggs reached the infective stage it was considered a good result. (Glaser and Stoll had found in their experiments that as many as 25 p.c. of the viable eggs of *Haemonchus contortus* reached the third stage). Some of this variation in results may be explained by the fact that the medium in different tubes varied greatly in consistency. This, in turn, was probably due to the variation in size of the piece of kidney added, the fluid from which would dilute the agar. Development seemed to be worse in the more fluid media; possibly the reducing action of the fresh kidney produces partially anaerobic conditions at the bottom of the tube and the larvae, which sink there unless the medium is fairly stiff, are then unable to develop.

Although the best results were obtained with the medium consisting of liver-extract agar plus ground yeast plus kidney the advantage was only slight and it was not certain that the addition of the ground yeast or the liver extract was of any real benefit.

It is a tedious and difficult job preparing a medium containing fresh sterile tissue. As well as the ever present risk of contamination with micro-organisms, in which case some or all of the batch is wasted, the final medium is complex and not suited for many physiological and biochemical studies. Hence an attempt was made to substitute kidney extracts for the fresh tissue. The kidney extracts were made up avoiding the use of heat as far as possible. Two parts by weight of tap water were added to one part of minced ox kidney; the whole was then stirred and allowed to infuse overnight at 4° C. Next morning the liquid was strained

off from the residue. Part was filtered through a Seitz filter after adjusting the pH to 7·0. The other part was heated to boiling to coagulate the protein, filtered through paper, the pH adjusted to 7·0 and then filtered through a Seitz filter. It will be referred to later as the "heated kidney infusion." The kidney residue that had been strained off was heated at 100° C. for 20 minutes with an equal weight of N/10 NaOH and the pH of the liquid was then brought to 7·0. After 30 minutes the pH was checked and the liquid strained off and rejected. The kidney residue was then spread on a filter paper to remove the last of the liquid before it was tubed and autoclaved at 10 lb. for 10 minutes. A series of media consisting of mixtures of the two kidney extracts, liver extract, peptone water and kidney residue was made up and dispensed into 5 inch × ½ inch test tubes to a height of about 1 cm. Ground heated yeast, as used by Glaser and Stoll, was added to some of the tubes of each kind of medium.

The basal medium consisted of one or the other of the two kidney extracts together with an equal volume of 1 p.c. liver extract (Eli Lilly), the whole made semi-stiff by the addition of one tenth volume of 2·5 p.c. agar. Larvae did not develop to the third stage in the medium. Apparently the water soluble extractives from kidney, even though not subjected to heat, cannot replace the fresh kidney tissues. However, the addition of kidney residue to the medium enabled some of the larvae to reach the third stage. It was also found that it was not necessary to sterilize the kidney extract by Seitz filtration; it could be autoclaved with the kidney residue. The liver extract seemed to be unnecessary (this Eli Lilly liver extract is said to be protein free) but the presence of peptone (1 or 2 p.c. of Difco Proteose Peptone was used) which had been introduced to replace some of the water soluble substances lost from the kidney, and perhaps also of ground yeast, seemed advantageous.

The results with these media were still not particularly good. Even in positive cultures few of the viable eggs added became third-stage larvae and some cultures failed completely. The medium giving the most consistently successful results was 2 p.c. peptone, heated-kidney-infusion agar plus kidney residue.

It was decided to try the effect of adding "Lab Lemco," a meat extract used in bacteriological media, and "Yeastrel," a yeast preparation of the type of "Marmite," in the hope of getting better results, while at the same time finding out whether there was any special virtue in kidney or if it could be replaced by muscle. The composition of the various media made up is shown below. The heated kidney infusion was made as before save that it was not passed through a Seitz filter, as the finished medium was to be autoclaved. The heated veal infusion was made up in a similar way. The other ingredients (Lab. Lemco, etc.) were then added and the pH adjusted to 7·1. The meat residue was added to the test tubes (5 inch × ½ inch) to a height of about 1 cm. and the rest of the medium pipetted on so as just to cover the meat. The concentration of agar was 0·25 p.c. All the media were autoclaved at 15 lb. pressure for 15 minutes.

(1) Kidney residue + water agar.
(2) Kidney residue + 2 p.c. peptone agar.
(3) Kidney residue + 2 p.c. peptone heated kidney infusion.
(4) Kidney residue + 2 p.c. peptone heated kidney infusion agar.
(5) Kidney residue + 2 p.c. peptone, 0·5 p.c. Lemco agar.
(6) Kidney residue + 2 p.c. peptone, 0·5 p.c. Lemco heated kidney infusion agar.
(7) Kidney residue + 2 p.c. peptone, 0·5 p.c. Lemco, 0·3 p.c. Yeastrel heated kidney infusion agar.
(8) Kidney residue + 2 p.c. peptone, 0·5 p.c. liver extract heated kidney infusion agar.
(9) Veal residue + 2 p.c. peptone heated kidney infusion agar.
(10) Veal residue + 2 p.c. peptone heated veal infusion agar.

Third stage larvae developed on all these media save medium (1) and medium (5) though in the former one larva was seen in the transitional stage between second and third stage. These media contain no kidney infusion. The only other medium without kidney infusion (or veal infusion) was medium (2) and in this only one larva reached the third stage and it was markedly abnormal in morphology. In short, those media without a meat infusion were markedly inferior and "Lab. Lemco" could not make up the deficiency. The addition of "Lab. Lemco" and "Yeastrel" to the peptone kidney infusion agar did not improve the results. However, the addition of liver extract led to more consistent success, unlike the result of the previous experiment. Medium (3), which contained no agar, was quite successful whereas it had been found that fresh kidney tissue and nutrient broth would not allow development to the third stage. Possibly the fresh tissue had greater reducing powers and the semi-anaerobic conditions prevented development of the larvae. Media (9) and (10) showed that veal could replace the kidney, both as infusion and as residue.

It may be added that the kidney or veal residue can be replaced by Difco Veal powder or Difco Beef Heart powder. These are desiccated and powdered forms of meat made by the Difco Laboratories, Detroit, Michigan. They were added in a concentration of 5 p.c. to veal infusion prepared as before, warmed at 50° C. for 20 minutes and the pH adjusted to 7·0. The media were dispensed in 5 inch × ½ inch test tubes and autoclaved at 10 lb. for 10 minutes.

## DISCUSSION.

Although it was found that the free-living forms of *A. braziliense* could develop under aseptic conditions on a number of different media, the results were always poor. Only a small proportion of viable eggs ever reach the third stage, whereas the great majority reach this stage when living bacteria are present. Dead bacteria, however, seem to be of little use as a source of food, the larvae never reaching the third stage in this case. The living bacteria do not seem to produce any extra-cellular, heat-labile substance that promotes growth, as Seitz filtrates of cultures when added to killed bacteria do not permit development to take place. As the larvae actively ingest and presumably digest bacteria, there is, of course, no necessity for such a substance. Very little work has been done on digestion in parasitic nematodes (cf. review by Ackert and Whitlock), but it seems most likely that the digestion is extra-cellular, and hence, that autolysed micro-organisms might be able to replace the living bacteria. However, autolysed yeast proved ineffective.

Curiously enough, in spite of the striking difference between the growth-promoting properties of living and dead bacteria, just as good results were usually obtained with autoclaved kidney media as with those containing the fresh tissue. Some heat stable, water insoluble substance present in kidney or muscle seemed to be the important factor. Although the addition of ground, heated yeast to these media containing kidney was of doubtful benefit, it seems possible that sterile bacterial autolysates, prepared without heating at any stage, might contain the growth factors missing in meat and that a medium prepared on these lines might permit a greater proportion of larvae to reach the third stage.

## SUMMARY.

Although larvae of *A. braziliense* can develop to the third stage in suspensions of living bacteria, they cannot reach this stage in suspensions of dead bacteria (killed by heat, ultra-violet light or acetone) or in filtrates of bacterial cultures or in various combinations of these with nutrient broth and yeast and yeast extracts.

They can develop to the third stage under sterile conditions in a medium of fresh rabbit tissue and water agar. This medium is perhaps improved by the addition of liver extract and ground, killed yeast.

The most important constituent in this medium seems to be a heat stable, water insoluble factor, contained in kidney and veal; though a heat stable, water soluble factor contained in kidney or veal infusion is also necessary for good results.

Heat sterilized media seem as good as those containing fresh, sterile kidney.

None of the media permitting aseptic cultivation of the larvae gave as good results as those in which living bacteria were present.


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# PRODUCTION OF AMINOBENZOIC ACIDS BY BACTERIA RESISTANT TO SULPHONAMIDES

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After a number of workers, particularly Green (1940), had shown that extracts from yeast and some bacteria (Stamp, 1939) were antagonistic to the bacteriostatic action of sulphonamides, Woods found that p-aminobenzoic acid in very small concentration was capable of reversing the inhibitory action of sulphonamides on a number of bacteria, and showed that there was some evidence for the identity of this substance with the growth-stimulating factor of yeast and bacteria. McIntosh and Whitby (1939) had suggested that the action of sulphonamides in inhibiting bacterial growth was due to their interference with a particular enzyme system, and Woods and Fildes (1940) suggested that the enzyme concerned had p-aminobenzoic acid as its normal substrate. This view was further strengthened by the discovery that p-aminobenzoic acid was an essential metabolite for certain bacteria, by its isolation by Rubbo and Gillespie (1940) from the anti-sulphonamide extract of yeast and by the recent discovery that it is a constituent of folic acid. Woods and Fildes (1940) proposed that the action of sulphonamides was due to their structural similarity to p-aminobenzoic acid and their competition with this substance for the enzyme.

Although the theory has been challenged on a number of grounds by several workers, including Henry in his comprehensive review (1943), it is to-day almost universally accepted.

One logical and attractive extension was the attempt to explain the phenomenon of drug-resistance, both natural and acquired, in terms of the production of p-aminobenzoic acid by the organism. Both Woods (1940) and Selbie (1940) suggested that the variation in susceptibility of different organisms might be due to variation in the amount of p-aminobenzoic acid normally produced, while Green (1940) in his somewhat earlier paper inclined to the view that the rate of liberation of the growth-stimulating substance was the factor responsible.

A number of workers have endeavoured to produce experimental evidence to decide this important point. Obviously the establishment of the correctness

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of the view rests on the demonstration of an increased rate of production, or an increased total production of p-aminobenzoic acid by a resistant strain as contrasted with the susceptible parent strain, and the proportionality of this increase to the degree of sulphonamide resistance.

From the work of MacLeod, Mirick, Green and Bielschowsky, Spink and Vivino, and others, there can be no doubt that with resistant strains of many organisms there is a great increase in production of a sulphonamide antagonist. The identity of the substance with p-aminobenzoic acid is, however, open to question. MacLeod (1940) pointed out that there were a number of important differences between the sulphonamide inhibitor in his organisms and p-aminobenzoic acid, particularly in regard to ether-solubility; differences have also been noticed by other workers.

Landy *et al.* (1942, 1943), sought more accurate information by using a more specific test for p-aminobenzoic acid, based on the indispensability of this substance for the growth of *Acetobacter suboxydans*. In this way they demonstrated that a resistant strain of *Staphylococcus aureus* produced seventy times as much of the growth-promoting substance as did the parent strain.

Green and Bielschowsky (1942) likewise showed an increase in production of sulphonamide-antagonist with development of sulphonamide-fastness, but could not show any correlation between the yield of this substance and the degree of resistance.

Reviewing these results, Henry (1943) pointed out that no satisfactory proof for the hypothesis had been put forward.

The main difficulty is in the lack of specific methods for the isolation of p-aminobenzoic acid from the bacterial cultures. Practically all of the earlier workers used methods quite unspecific for p-aminobenzoic acid; the nearest approach to a specific method was that of Landy *et al.* (1943), (cf. above); anthranilic acid, for instance, is no growth factor of the Acetobacter.

Rubbo and Gillespie (1940), and Blanchard (1940) isolated from the growth-promoting, antisulphonamide extracts of yeast a substance which was shown quite unequivocally to be p-aminobenzoic acid. Similar methods applied to bacteria made resistant to sulphonamides have not so far been reported. Attempts have therefore been made to develop a method of extraction and separation of aromatic amino-acids, which would be sufficiently sensitive to be used with the low concentrations of these substances found in bacterial cultures.

The method adopted consisted in the transformation of the aromatic amino-acids into azo-dyes by diazotization and coupling with dimethyl-α-naphthyl-amine, in the separation of the acidic from the non-acidic dyes and in separation of the former by chromatography on alumina.

A preliminary account of this work has been given by Lemberg, Tandy and Goldsworthy (1945).

## PART I. BACTERIOLOGY. MATERIALS AND METHODS.

*The organism*: In February, 1945, a strain of *Bacterium coli commune* was obtained from the Commonwealth Serum Laboratories, Melbourne, (C.S.L.). Although the culture was pure in the generally accepted sense of the word, it was plated out and a single colony subcultured into broth for further tests. A stained film from this broth culture (24-hours-old) showed Gram-negative rods decidedly longer than usual and stained somewhat irregularly. In view of the source of the culture and its apparent conformity to the description of *Bact. coli commune*, fermentation tests were deferred and the experiments described below were begun with this single-colony subculture; it was stored in meat medium for purposes of maintenance and future reference, as was also the original strain from the C.S.L.

Subsequent tests in May, 1945, on the stock culture and on the organism after serial passage in the experimental media (Kisch) showed clearly that we were working with a non gas-producing variant and that only a few organisms per centum of the original C.S.L. strain seemed capable of producing gas, which explains how we came to select the variant used in this work.

The characters of the organism used throughout these experiments are described here: *Colonial*: the colonies were generally large and flat, with a rough or matt surface which showed numerous facets, somewhat as seen in Fig. 123 in the second edition of Topley and Wilson's book "Principles of Bacteriology and Immunity." *Microscopical*: The organism appeared as a long Gram-negative rod, almost filamentous; motility was doubtful. *Biochemical*: Glucose, lactose, maltose, dulcitol and mannitol were fermented with the production of acid but not gas; sucrose and salicin were not fermented; indole was readily produced; the methyl red test was positive and the Voges-Proskauer test negative.

*The inoculum*[2]: Under the experimental conditions it was found that the relatively small inoculum of 2,000 organisms per 2 ml. (final concentration in the experimental medium) could be safely used. This number was repeatedly checked by plate-counts made on the actual material used in the daily (or two-daily) passage of the organism in the experimental media.

*The medium*: Kisch (1919) has described a useful synthetic or semi-synthetic medium (p. 32 *loc. cit.*) which we found quite satisfactory. Two thousand organisms per two ml. grow freely to at least $100 \times 10^6$ viable organisms per ml. within 24 hours. This medium was, therefore, used as a basal medium to which any desired concentration of drug was added.

*The drug*: Sodium sulphathiazole was selected and an anhydrous preparation used. At intervals of about six weeks a concentrated aqueous solution containing various amounts of the drug (according to requirements) was prepared and stored in the refrigerator as a stock solution. This aqueous solution was diluted in Kisch medium to a convenient concentration so that in any experiment the Kisch medium was not unduly diluted by the addition of water from the aqueous solution of drug. The addition of drug to Kisch medium never lowered the concentration of the constituents of the medium by more than 10 p.c., whereas we repeatedly found that full growth could be obtained when the basal medium was diluted 30–40 p.c. by the addition of water (see below, series (a) ). The pH of the medium was not significantly altered by the amounts of drug solution added to it.

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2 The desirability of using a small inoculum should be emphasized. It is well-known that an otherwise poor or even inhibitory medium may be converted to one at least tolerable to a given organism by the introduction of a large mass of bacterial bodies or products. It would be reasonable, therefore, to assume that, other factors being held constant, acclimatization of an organism to an inhibitory substance will be more faithfully indicated when a small inoculum is used rather than a large one. This would, in part, explain apparent variations in the success attending the efforts of different workers to produce strains of bacteria with very high resistance to bacteriostatic agents.

*Technique*: In outline the procedure was as follows:

1. The organism was subcultured in the Kisch medium three times at intervals of 24 hours to eliminate all traces of the original broth medium (whose concentration after the three passages was only 1 part in $10^{15}$) and to allow the organism time to accustom itself to the less favourable medium.

2. Thenceforward, three principal series of passages were run: (a) in Kisch medium diluted with water; (b) in Kisch medium containing added solution of drug, the interval between transfers of the organism being 24 hours; (c) in Kisch medium containing added solution of drug, the interval between transfers being 48 hours. A fourth series was also run in Kisch medium unchanged by any additions.

3. After various numbers of passages the organisms from the different series were grown in the basal medium for purposes of biochemical investigation.

For general biological reasons it was decided to keep the number of generations of the organism in all series as nearly equal as possible. To this end each of the three principal series of passages was continued by selecting the inoculum according to a "titration" experiment (Table 1).

TABLE 1.

| Tube No. | Kisch medium ml. | Drug soln. ml. | Drug conc. mg. p.c. | Degrees of opacity due to density or bacterial population. 24 hrs. | 30 hrs. | 47 hrs. |
|---|---|---|---|---|---|---|
| 1 | 1·9 | 0·1 | 1 | 3 | 3 | 3 |
| 3 | 1·7 | 0·3 | 3 | 3 | 3 | 3 |
| 5 | 1·5 | 0·5 | 5 | 3 | 3 | 3 |
| 7 | 1·3 | 0·7 | 7 | 2 | 3 | 3 |
| 9 | 1·1 | 0·9 | 9 | trace | 3 | 2½ |
| 11 | 0·9 | 1·1 | 11 | — | 2 | 2½ |
| 13 | 0·7 | 1·3 | 13 | — | — | 2½ |
| 15 | 0·5 | 1·5 | 15 | — | — | 2½ |
| 17 | 0·3 | 1·7 | 17 | — | — | 2½ |
| 19 | 0·1 | 1·9 | 19 | — | — | ½ |
| Control | 2·0 | 0·0 | 0 | 3 | 3 | 3 |
| Blank | 2·0 | 0·0 | 0 | — | — | — |

Note: Drug solution contains (say) 20 mg. p.c. in Kisch medium. Inoculum: All tubes (except Blank) received 2,000 organisms suspended in 0·1 ml. Kisch medium (this extra volume has been ignored in making any calculations).

The figures in the last three columns indicate the amount of growth, using Control as standard. Full growth was assumed to have occurred when the opacity corresponded to 2½ or 3.

In the example given, tube 5 would be selected as that in which in the time allowed (here 24 hours) maximum growth had just been attained, so that the phase of decline in the number of viable organisms would not vitiate an estimate based on opacity and on a calibration of opacity by means of plate counts. From this tube would then be prepared the dilute suspension to be used for the inoculation of the next "titration," and so on.

Periodically the cultures of the organisms in the various series were tested for purity and for constancy of character.

## PART II. BIOCHEMISTRY.

### A. METHODS.

The supernatant from a centrifuged culture of the organism was acidified with hydrochloric acid, the aromatic amines diazotized with sodium nitrite, the excess of the latter destroyed with ammonium sulphamate, and the diazonium salts so produced coupled with either dimethyl-α-naphthylamine (D.M.N.) or N (1-naphthyl) ethylene diamine dihydrochloride (N.E.D.), the reagent of Bratton

TABLE 2.

*Extinction coefficient of dyes from p-aminobenzoic and anthranilic acid.*

| Substance | | P.A.B.A. | | Anthranilic acid | | |
|---|---|---|---|---|---|---|
| Coupling Component | | N.E.D. | D.M.N. | N.E.D. | D.M.N. | |
| Coupling Method | | (I) | (I) | (I) | (II) | (III) |
| Technique | Final HCl conc. p.c. | $\epsilon$540 | $\epsilon$540 | $\epsilon$540 | $\epsilon$540 | $\epsilon$540 |
| A | 0·5 | 0·356 | 0·353 | 0·188 | 0·170 | — |
| | 1·0 | 0·369 | 0·322 | 0·158 | 0·149 | — |
| | 2·0 | 0·366 | 0·253 | 0·107 | 0·142 | — |
| | 5·0 | 0·355 | 0·128 | 0·051 | 0·120 | — |
| B | 0·5 | 0·358 | 0·338* | 0·265 | 0·154 | 0·169 |
| | 1·0 | 0·354 | 0·305 | 0·231 | 0·159 | 0·197 |
| | 2·0 | 0·362 | 0·227 | 0·152 | 0·165 | 0·194 |
| | 5·0 | 0·359 | 0·107 | 0·076 | 0·133 | 0·201 |

* With coupling method III; 0·372.

Notes.

Technique A. 1 ml. solution + x ml. 10 p.c. HCl + (6 — x) ml. $H_2O$ + 1 ml. 0·1 p.c. $NaNO_2$ at room temperature.

Allow to stand 3 minutes. Then add 1 ml. 0·5 p.c. ammonium sulphamate. Allow to stand 2 minutes. Then add 1 ml. coupling reagent.

Technique B. As A, but omitting 3 minutes' standing after addition of nitrite.

Coupling Component. N.E.D.: 0·1 p.c. aqueous solution of N (1-naphthyl) ethylene diamine dihydrochloride.

D.M.N.: 1·0 p.c. solution of dimethyl-α-naphthylamine in alcohol.

Coupling Method. (I) Allow to stand for 1 hour.

(II) Allow to stand 1 hour, then neutralize to faint cloudiness with saturated sodium acetate solution, allow to stand 1 hour, reacidify, and dilute to 20 ml.

(III) As (II), but neutralize as soon as prepared and allow to stand for 16 hours.

and Marshall (1939). The former coupling component produced ether-soluble dyes, and was used for the purposes of extraction and separation; the latter gave more rapid and complete coupling, and was therefore better suited for estimation of the total quantities of diazotizable amino-compounds present. Early experiments on the supernatants showed us that among the substances present were p-aminobenzoic acid and anthranilic acid. Consequently, detailed investigation of these was carried out.

*Conditions of Diazotization and Coupling.*

Table 2 shows the effects of different techniques of diazotization (cf. A and B, Table 2), of different concentrations of hydrochloric acid, and of different coupling components on the extinction coefficients[3] of the resulting dye solution, using solutions of p-aminobenzoic acid (P.A.B.A.) and anthranilic acid (o-aminobenzoic acid) of initial strength 10 $\mu$g./ml.

For estimation of total diazotizable amines, technique B with a final HCl concentration of 0·5 p.c., and with N.E.D. as coupling component, gave the most satisfactory results, assuming that P.A.B.A. and anthranilic acid provided the major part of the amines present.

For isolation of amines, where D.M.N. must be used, technique B was again most favourable. A concentration of hydrochloric acid of about 1 p.c. was found

TABLE 3.

*Specific coefficients of dyes from amino compounds.*

| Substance. | Method. | N.E.D. | | D.M.N. | |
|---|---|---|---|---|---|
| | | $\epsilon$sp of dye. | $\epsilon$sp of amino acid as dye. | $\epsilon$sp of dye. | $\epsilon$sp of amino acid as dye. |
| P.A.B.A. | 1 | 154 | 360 | 150 | 350 |
| | 2 | 131 | 292 | 64 | 144 |
| | 3 | — | — | 116 | 270 |
| Anthranilic acid | 1 | 120 | 280 | 86 | 200 |
| | 2 | — | — | — | — |
| | 3 | — | — | 116 | 270 |

Notes.

Methods: 1. Solution of pure crystalline substance diazotized and coupled by method B(I) with N.E.D. and B(III) with D.M.N. (see Table 2) in 0·5 p.c. HCl. Final concentration of substance, 1 $\mu$g./ml.

2. As method, but final concentration of substance, 10 $\mu$g./ml.

3. Weighed amount (about 1 mg.) of pure crystalline dye. dissolved in 5 ml. alcohol, and diluted to 100 ml., with 2 p.c. HCl.

[3] The instrument used throughout this work was a Hilger-Nutting visual spectrophotometer, the property of the National Health and Medical Research Council.

satisfactory for diazotization, provided that after the addition of the dimethyl naphthylamine, sodium acetate solution was added until a faint cloudiness appeared.

Differential estimation of P.A.B.A. and anthranilic acid should be possible by combining two techniques, e.g. A (I), 5 p.c. HCl, N.E.D.; and B (I), 0·5 p.c. HCl, N.E.D. This was found to be so, provided the concentration of the two substances fell in the range of ratios 3 : 1 to 1 : 3. However, since the ratio is not in this range and since other diazotizable amines of unknown behaviour under various diazotization conditions are probably also present in the supernatants of the bacteria, the technique could not be used.

Dyes from P.A.B.A. were relatively insoluble, and in high concentration gave lower extinction coefficients than normal. The pure crystalline dye evidently did not give a true solution in alcoholic HCl (cf. results of method 3 with those of method 1, Table 3).

Anthranilic acid was not completely converted to the dye by diazotization and coupling with D.M.N. Hence, solutions of the pure crystalline dye, which was readily soluble, gave higher extinction figures than the solutions prepared from the pure crystallized acid by diazotization and coupling.

For estimation of total diazotizable amines in supernatants, an average specific extinction coefficient of 300 was used, since anthranilic acid was present in greater concentration than p-aminobenzoic acid.

*Properties of Azo-dyes.*

*Dye prepared from P.A.B.A. with dimethyl-α-naphthylamine.* After diazotization and coupling, the dye was extracted with ether and the ether solutions re-extracted with 2 p.c. sodium carbonate solution, leaving the excess dimethylnaphthylamine in the ether. The dye was then brought back into ether by gradual neutralization with dilute phosphoric acid.

The chromatography was carried out on Merck's "Brockmann" alumina washed previously with 5 p.c. hydrochloric acid, then with large amounts of water and finally dried at 150° C. The dye was adsorbed from its ethereal solution (dried with anhydrous sodium sulphate) on top of the column as an orange band. On development with 2 p.c. acetic acid in ether this band passes down the column and finally leaves it as yellow solution. Only a trace remains adsorbed at the top of the column.

Neutral dye: From petroleum ether glittering red-brown leaflets with strong pleochroism and oblique extinction; from sodium carbonate solution by neutralization with acetic acid in short brick-red needles; m.p., on the copper block, 229–230° C. (corr.).

The dye could be extracted from ether by 1 p.c. sodium carbonate or 20 p.c. hydrochloric acid, less completely by 10 p.c. HCl and little by sodium bicarbonate solution.

From the violet solution in HCl the hydrochloride precipitated as fine straight blue needles with green lustre. The hydrochloride was practically insoluble in hot 10 p.c. hydrochloric acid. It crystallized rapidly in the same form as above from either alcohol or acetic acid on addition of 10 p.c. HCl. The very characteristic crystals showed no pleochroism.

*Dye obtained from m-aminobenzoic acid with dimethyl-α-naphthylamine.* The dye resembled that of P.A.B.A. far more than that of anthranilic acid. The neutral dye crystallized from petroleum ether as concentric orange clusters of needles or flat prisms without marked pleochroism. M.p. 198° C. (corr.). The hydrochloride could be recrystallized from hot 10 p.c.

hydrochloric acid, from which it came out much more slowly than the hydrochloride of the P.A.B.A. dye, in the form of brownish-purple needles with straight ends and strong pleochroism.

*Dyes prepared from anthranilic acid with dimethyl-α-naphthylamine.* The crude dye crystallized directly from the neutralized solution after diazotization and coupling in the form of bluish-violet needles with green sheen. It could be recrystallized from glacial acetic acid or ether by the addition of water or petroleum ether respectively. The m.p. was found to vary between 130° C. and 153–154° C. (corr.).

The solution in ether was of orange-pink colour; whereas the P.A.B.A. dye did not readily pass from ether into water, washing of the ethereal solution of the anthranilic acid dye always removed a fraction with blue-violet colour. The solution of the dye in acetic acid is of the same colour. Only the o-aminobenzoic acid dye exists in such a blue-violet form. Its behaviour towards sodium bicarbonate, sodium carbonate and hydrochloric acid, however, did not differ from that of the P.A.B.A. dye, and the solutions were of the same colour.

The hydrochloride was more difficult to crystallize, but was obtained in long straight square-ended needles with slightly oblique pleochroism.

In the chromatogram of the ethereal solution on the acid-washed alumina the anthranilic acid dye was adsorbed on the top of the column as a dark brown band. 2 p.c. acetic acid in ether changed the colour of the band to a brilliant blue-violet, but moved the band little. On further development with 20 p.c. acetic acid in ether the band divided, one violet band moving down the column and being finally eluted with orange colour, while the remainder stayed at the top of the column. The first band yielded a pigment of the same melting point and behaviour as the crude dye. The top band was removed by elution with glacial acetic. After addition of ether and removal of the acetic acid by repeated washing with water and dilute sodium bicarbonate solution, a red ethereal solution was obtained which on evaporation to a small volume yielded prismatic crystals with a golden-bronze lustre of m.p. 236–238° C. (corr.).

On standing in solution in 10 p.c. ammonia or in 20 p.c. hydrochloric acid this red form was partly reconverted into the low melting range form, the chromatogram of the ethereal solution again separating the two dyes.

The fraction of the anthranilic acid dye yielding the red form varied from 30–70 p.c., diazotization and coupling in very dilute solution yielding more of the red form.

*Chromatographic Separation of the P.A.B.A. and Anthranilic Acid Dyes.*

The above results indicated that chromatographic separation of these dyes was possible using various strengths of acetic acid in ether for development. From ether both dyes were adsorbed on the acid-washed alumina as a band at the top of the column. Development with 2 p.c. acetic acid in ether caused the P.A.B.A. dye to precede the violet band of the anthranilic dye in the form of a yellow band. On increasing the concentration of acetic acid to 20 p.c., a part of the anthranilic acid dye passed through and yielded an orange solution, leaving the second purple band at the top of the column as before.

It is important to note that different preparations of alumina give quite different results. For example, on Hopkin and William's alumina the anthranilic acid dye gave a reddish-orange band, which preceded the yellow band of the P.A.B.A. dye, even with 0·5 p.c. acetic acid in ether, leaving the column colourless, while a batch of American Merck's alumina (which had evidently been activated with alkali) generated bubbles of carbon dioxide which disrupted the column during the washing with the acetic-ether. After preliminary washing with 2 p.c.

acetic in ether, followed by washing with ether, the dyes were adsorbed as normally on the top of the column, but 2 p.c. acetic acid in ether gave two bands from the anthranilic acid dye, one of which preceded that of the P.A.B.A. dye. The conditions of chromatography must therefore be determined for the particular batch of alumina used, and not all preparations are equally suitable.

Recovery experiments gave the following results: P.A.B.A. alone, in a concentration of 0·1 to 1·0 mg. per litre yielded the dye quantitatively under the conditions of diazotization and coupling applied to the study of the supernatants. On chromatography 87 p.c. of the dye was recovered in the 2 p.c. acetic acid-ether solution passing the column.

Anthranilic acid, in the same concentrations, gave a somewhat variable yield of the dye (40–75 p.c. of theory), while after chromatography 75 p.c. of this could be eluted from the column. The overall yield varied between 30 and 50 p.c.

In order to test the separation of the dyes under conditions close to those attaining in the supernatants, a mixture of 0·12 mg. P.A.B.A. and 4·5 mg. anthranilic acid in 5 litres of N/5 hydrochloric acid was used.

Assuming, as in the experiments above, $\epsilon^{540}_{sp.}=350$ for P.A.B.A. and $\epsilon^{540}_{sp.}=270$ for anthranilic acid in hydrochloric acid, 42 p.c. of the dyes was recovered in the ether solution. No loss was experienced during the extraction of the dyes from ether by sodium carbonate and their re-extraction from the acidified solution by ether. After chromatography 88 p.c. of P.A.B.A. and 30 p.c. of anthranilic were recovered in the respective fractions.

As will be seen from Table 5, the recoveries from the bacterial supernatants were somewhat smaller, both in the diazotization and coupling and in the chromatography. The loss on coupling with D.M.N. is probably due to the presence of other diazotizable amines in the supernatants which couple less completely with dimethyl-α-naphthylamine than does diazotized anthranilic acid. There was also some loss during the extraction of the dyes from ether by sodium carbonate, indicating the presence of small amounts of non-acidic diazotizable amine. The larger loss in the chromatography may be largely due to the fact that in these experiments glacial acetic acid had been used as elucent of the anthranilic acid dye adsorbed at the top of the alumina column, while in the recovery experiments this was eluted by ammonia. The latter reagent leaves the alumina practically colourless, while the alumina eluted by acetic acid remains slightly purple.

In order to be on the safe side a recovery of 33 p.c. has been assumed. This is approximately correct for anthranilic acid, while the actual amounts of P.A.B.A. present are certainly smaller than those calculated in this way.

### B. INVESTIGATION OF PRODUCT OF B.C.C.

On the basis of the findings reported above with dyes prepared from p-aminobenzoic acid and anthranilic acid, a number of investigations of the supernatant

fluids from centrifuged cultures of *Bact. coli commune* were undertaken. The supernatants were obtained at various times during the passaging reported in the first part of the paper.

The experiments took three main forms:

(1) Small-scale estimations of total diazotizable amines in supernatants from cultures of normal and drug-adapted organisms, and the appearance of the amines with the ageing of the culture.

(2) Medium-scale extractions of diazotizable amines on a quantitative basis, with subsequent separation of various fractions by extraction of the acidic dyes from ether, followed by chromatography.

(3) A large-scale extraction of the amines from 25 litres of supernatant, with a view to crystallizing and identifying the dyes.

The following facts were established by preliminary experiments:

(1) Uninoculated Kisch medium contained no diazotizable substances;

(2) P.A.B.A. and anthranilic acid were not destroyed by boiling or autoclaving for one hour;

(3) Under conditions of the experiment, the traces of dye from the sulphathiazole transferred with the inoculum to the drug-free media under investigation, could not be detected in the ether solution of amines obtained, and were too small to affect quantitative estimates of the amino-compounds produced by the organism;

(4) When the supernatant to be investigated contained drug, the dye from this drug was readily separable from those of P.A.B.A. and anthranilic acid.

*Appearance of Diazotizable Amines with Ageing of the Culture.*

Four sets of experiments were carried out, the results being shown in Table 4. In each experiment a litre of drug-free medium was inoculated with the drug adapted organisms, as described in Part 1, and daily samples were removed for estimations of the amino-compounds developed. Experiments with this technique are labelled "a" in Table 4.

In three cases, on the first day of growth of such an inoculated flask, the organism was subcultured into 10 ml. of Kisch medium in a test tube. On the following day, this tube-culture was subcultured into a second tube, and then the residual culture (supernatant) in the first tube was analysed for amino-compounds. This procedure was repeated daily. In this way, a series of cultures were obtained in which the organism was growing in the absence of drug, but was maintained alive for the whole period of the experiment; whereas in the flasks there were usually no viable organisms after four or five days. Experiments of this type are labelled "b" in the Table. They showed no significant difference from the analagous cultures which were continuously incubated for six days.

In Table 4, the figures for "amount of drug tolerated" are the concentrations of sodium sulphathiazole to which the organism was resistant (see Part 1) immediately before use for

TABLE 4.

| Organism. | Unadapted in Kisch-Water. | | | | | Adapted, 24-hour series. | | | | Adapted, 48-hour series. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. of passages | 40 | 56 | 140 | | 200 | 47 | 142 | | 200 | 24 | 62 | | 100 |
| Amount of drug tolerated† | 0 | 0 | 0 | | 0 | 0·5 | 3 | | 4 | 2·0 | 24 | | 25 |
| Day of growth | mg./l* | mg./l* | mg./l* | | mg./l* | mg./l* | mg./l* | | mg./l* | mg./l* | mg./l* | | mg./l* |
| | a | a | a | b | a | a | a | b | a | a | a | b | a |
| 1st | 0 | 0 | tr | — | 0 | 1·05 | tr | — | tr | 0·0 | tr | | 1·20 |
| 2nd | 0 | 0 | tr | 0 | 0 | 1·10 | tr | tr | tr | 0·9 | 0·90 | tr | 1·25 |
| 3rd | 0 | 0 | 0 | 0 | 0 | 1·00 | tr | tr | tr | 0·7 | 0·85 | 0·75 | 1·25 |
| 4th | 0·9 | tr | 0 | 0 | 0 | 1·15 | tr | tr | tr | 0·9 | 0·75 | 0·90 | 1·25 |
| 5th | 0·85 | 0 | 0 | 0 | 0 | 1·05 | tr | tr | tr | 0·75 | 0·75 | 0·80 | 1·25 |
| 6th | 1·10 | 0 | 0 | 0 | 0 | 1·50 | tr | tr | tr | 0·95 | 0·80 | 0·70 | 1·25 |
| 7th | 0·75 | | | | | 0·85 | | | | | | | |
| 8th | 0·85 | | | | | 1·25 | | | | | | | |
| 9th | 0·95 | | | | | 1·29 | | | | | | | |
| Culture sterile on | ? | 4th day | 5th day | | 4th day | ? | 5th day | | 4th day | ? 4th day | 5th day | | 4th day |

a and b, cf., p. 18. tr = trace. † = mg. per 100 ml.

* = amino-compounds mg./l, assuming *esp* = 300 and molecular weight of dye 319 (as in P.A.B.A. dye).

the present experiments. No experiments on the appearance of amino compounds with ageing of the culture were carried out in the presence of drug.

*Extraction and Quantitative Separation of Amines.*

(a) *Partition of amines between cells and medium.* A one-litre culture of the *unadapted* organism after 130 passages in Kisch medium was incubated for 24 hours at 37° C. It was then divided into two portions and centrifuged at 2,500 r.p.m. for ½ hour. The supernatant fluid from the two portions was removed and combined.

The deposits of cells were treated as follows: One sample was extracted with 50 ml. N/25 ammonium hydroxide solution, and the mixture was then centrifuged; after acidification the supernatant fluid was tested for diazotizable amines. No colour whatever was developed if the extract was boiled with diluted hydrochloric acid, diazotized and coupled. MacLeod (1940) had found that the antisulphonamide substance of *B. coli* grown in synthetic medium is not present in the supernatant, but can be extracted from the cells by N/25 ammonium hydroxide. Our findings show that this substance cannot be p-aminobenzoic acid.

The other sample of cells was lysed by boiling with 50 ml. distilled water until the volume fell to 20 ml.; then this solution was centrifuged and acidified. Diazotization and coupling with N(1-naphthyl) ethylenediamine dihydrochloride produced no colour.

The supernatant was examined for the presence of diazotizable amino compounds by the method described below [sect. (b)]. The total content of amines in 1 litre of supernatant was 3·3 $\mu$g.

The above procedure was repeated on 48- and 72-hour cultures of the same organism, with similar results. No amines could be detected in the bacterial cells, while the supernatants showed 3·7 and 2·5 $\mu$g. per litre respectively.

Similar experiments on 48-hour cultures of the 24-hour series drug-adapted organism after about 130 passages and of the 48-hour series drug-adapted organism after about 60 passages, gave similar results, the amine concentration in the medium being higher, particularly in the latter case. No amines were detected in the bacterial cells.

(b) *Extraction and separation of amines from supernatants*: Several experiments of this nature were performed on samples of approximately 1–2 litres.

The supernatant was acidified by addition of 1/50 vol. conc. HCl, and then boiled for 1 hour. (Later samples were not boiled as it was found that boiling had little effect). After cooling the amines were diazotized by addition of 20 ml. 1·0 p.c. sodium nitrite solution per litre, followed (after 3 minutes in early experiments, immediately in later ones) by the addition of 20 ml. of 5 p.c. ammonium sulphamate solution. Then after 2 minutes, 1 ml. per litre of dimethyl-α-naphthylamine was added, diluted, before addition, to a 10 p.c. solution in alcohol. The mixture was neutralized with saturated sodium acetate solution until faintly cloudy (due to a partial separation of the dimethyl-α-naphthylamine) and allowed to stand overnight. It was then neutralized further with sodium acetate solution, and extracted at least twice with ether. Extraction of the ether with 1 p.c. sodium carbonate solution removed the acidic dyes, leaving the unchanged dimethyl-α-naphthylamine and non-acidic yellow dyes in the ether. The carbonate solution was neutralized with phosphoric acid, and the dye shaken out with fresh ether. The ether solution was washed with water, dried with anhydrous sodium sulphate, concentrated and chromatographed. In every case, whether the organism used was adapted to sulphathiazole or not, the ether solution was of a brighter red colour than either P.A.B.A. dye (yellow) or anthranilic acid dye (orange-pink) solutions.

Chromatography on alumina gave qualitatively the same result in all experiments, differences between cultures being quantitative only. The whole of the dye was first adsorbed to the top of the column, the ether coming through colour-

less. Development with 1 p.c. acetic acid in ether separated out a faint, narrow, yellow band which moved through the column relatively fast, giving a yellow solution A. A blue-violet band also separated from the top band, and moved down slowly. It was removed from the column with 10–20 p.c. acetic acid in ether, giving an orange-red solution B and leaving a strong red-violet band at the top of the column. The latter was finally brought into solution by removing the alumina from the column and shaking repeatedly with glacial acetic acid, giving a red solution C.

*Solution A*: This dye behaved similarly to p-aminobenzoic acid dye, but could not be crystallized, because of the small quantity obtained.

*Solution B*: The ether extract was washed free of acetic acid, and the dye extracted from it with a small amount of 10 p.c. hydrochloric acid. On neutralization of this solution with sodium acetate, the colour changed from red-violet to blue-violet, a reaction given by the anthranilic acid dye but not by P.A.B.A. dye. One sample of this dye was successfully crystallized as long blue-violet needles, with slightly oblique pleochroism, identical in appearance

TABLE 5.

| Organism. | Unadapted. | | | Adapted. 24 hour series. | | 48 hour series. | |
|---|---|---|---|---|---|---|---|
| No. of passages | 0 | 90 | 140 | 20 | 135 | 24 | 62 |
| Drug tolerated, mg. p.c. | 0 | 0 | 0 | 0·3 | 3·0 | 2·0 | 24 |
| Vol. of supernatant, ml. | 1,880 | 1,950 | 880 | 1,990 | 890 | 1,840 | 900 |
| Total amines, μg. | 1,750 | 2,030 | ‡ | 1,100 | ‡ | 1,420 | 810 |
| Total ether extract, μg. | 680 | 550 | — | 190 | — | 500 | — |
| Final ether extract, μg. | — | 540 | 3·8 | 170 | 6·7 | — | — |
| P.A.B.A., μg., | ‡ | ‡ | ‡ | 8·3 | ‡ | 22 | 9 |
| Anthranilic, μg. | 381 | — | 2·5 | — | 4·0 | 210* | 146 |
| *S.A.T.* total amine | | | | 2·7 | — | 14 | 143 |
| *S.A.T.* P.A.B.A.† | | | | 120 | 1,800 abt. | 300 | 4,300 |
| *S.A.T.* O.A.B.A.† | | | | — | 1,200 | — | 27 |

* 20 p.c. acetic in ether-eluate only. Residual material on column not measured.

† Assuming 33 p.c. recovery of P.A.B.A. and anthranilic acid. The other values have not been corrected for losses.

‡ Too low to measure.

— Not measured.

with the synthetic dye from anthranilic acid. Melting-point determinations were not possible because of the minute amount of material obtained.

*Solution C*: The solution was diluted with ether, and neutralized with sodium bicarbonate, giving a deep orange-red solution. On extraction with 10 p.c. HCl, the whole of the dye passed into the acid. From the solution in 10 p.c. HCl the hydrochloride of the dye crystallized readily in fine, bent, purplish needles, with parallel extinction. As reported below, this dye is identical with the second dye from anthranilic acid, and has been included as such in Table 5. Table 5 shows typical quantitative results (so far as the quantities available permitted estimation) of investigations of this type.

In all the experiments tabulated in Table 5 the final culture for analysis had been made in drug-free medium in order to avoid the complication of large amounts of azo-dye being formed from the drug. Later it was found that the major part of the sulphathiazole dye hydrochloride could be removed by filtration, while the rest remained in the ethereal layer when the dyes of the amino acids were extracted from ether by sodium carbonate solution. Although at that time the main body of experiments had been completed and the adapted strain had been discarded, it was considered desirable to establish whether any change in the production of aromatic amino acids occurred on transferring the adapted organism from drug-containing to drug-free medium. The unadapted organism had been stored *in vacuo* for some months, and was once more adapted to sulphathiazole. The newly adapted organism differed from the previously adapted one in producing no anthranilic acid, while P.A.B.A. was produced in the same small concentrations as in previous experiments. This shows that the second drug-adapted strain differed from the first. The presence or absence of sulphathiazole in the culture medium did not influence the amount of P.A.B.A. found in the supernatant.

(c) *Bulk preparation*: An attempt was made by procedures similar to those outlined in (b) to isolate sufficient of the dyes for crystallization and identification.

Two 10-litre batches of culture medium were inoculated with the 48-hour series drug-adapted organism, and incubated for 48 hours. The organism at this stage had gone through 82 passages in the presence of sulphathiazole and tolerated a concentration of 15 mg. per 100 ml. It was subcultured twice in drug-free medium before being inoculated into the 10-litre batches.

Estimation showed 0·9 mg. total diazotizable amines per litre, or a total of 18 mg. amine in the whole batch (20 litres of supernatant).

After chromatography, the anthranilic acid dye fraction B corresponded to 0·7 mg. pure acid (not corrected for losses). The P.A.B.A. fraction A was not estimated quantitatively but was much less than this. Neither solution gave crystalline material.

Fraction C yielded approximately 3 mg. pure neutral dye in the form of thick lustrous, prismatic crystals, with a beautiful golden-bronze appearance by reflected light. M.p. (uncorrected) 220° C. Microanalysis gave the following result:

C 70·32 p.c.; H 5·58 p.c.; N 14·25 p.c.; O (by difference) 9·85 p.c.; calculated for $C_{19}H_{17}O_2N3$:

C 71·44; H 5·37; N 13·17; O 10·03.

In spite of the somewhat too low carbon and too high nitrogen values the analysis is in agreement with the assumption that the dye is derived from anthranilic acid (cf., p. 8).

## DISCUSSION.

As frequently occurs in work of this character, a number of unforeseen factors became evident only as the work progressed. It was not possible to obtain as complete sets of quantitative results as might perhaps be desired, because the metabolism of the organisms changed slowly throughout the period covered by the experiments and earlier stages of the work could not be repeated to make available data which later work showed desirable. However, the results are sufficient to make a number of points quite clear.

(1) Nature of amino compounds produced by B.C.C.: Unadapted organisms at the beginning of the experiments were found to produce, in the absence of sulphonamides, two aromatic amino acids capable of isolation by our procedure. These have been identified beyond reasonable doubt as anthranilic acid and p-aminobenzoic acid. Organisms adapted to sulphathiazole produced the same compounds.

Other diazotizable amines appear also to be present in the supernatants. Some of these couple after diazotization still less readily with D.M.N. than anthranilic acid, while they couple with N.E.D.; others, present in small amounts, yield ether-soluble, non-acidic dyes.

(2) Relative amounts of the amines: In the main series of experiments p-aminobenzoic acid always constituted the smaller fraction of the ether-extractable acidic amines. Anthranilic acid constituted the remainder. A second drug-adapted strain of the same organism showed a metabolism, which was entirely different from that of the first drug-adapted strain, in that only P.A.B.A. was produced but in no greater concentration than before.

(3) Changes during passaging and with drug-adaptation: At the beginning of the experiments the unadapted organism produced a concentration of almost 2 mg. per litre of total diazotizable amines in the culture medium after one day's incubation, but after 40 passages this had dropped to half, which, furthermore, did not appear until the culture was four days old. After 56 passages this has fallen still further, only traces being found; and at 130–140 passages the ether-extracted amine totalled less than 4 $\mu$g. per litre.

A similar phenomenon occurred when the organism was adapted to the drug by 24-hour passaging. After 130 passages the ether-extractable amine production had fallen to about 6 $\mu$g. per litre of culture medium. However, with the 48 hour passage technique, the amount of amine produced had not fallen so much. Twenty-four passages had lowered the ether-extractable material to two-thirds of the amount produced by the unpassaged organism, while the total diazotizable amine had fallen to somewhat less than half. However, even 100 passages did not lower the total amine below about 1 mg. per litre (cf. Tables 4 and 5).

Table 5 shows that the organism has not produced any greater amount of total diazotizable amine or of p-aminobenzoic acid in response to the presence of sulphonamide.

(4) P.A.B.A./S.T. ratios: By experiments on unadapted organisms, the ratio of P.A.B.A. to sulphathiazole required to reverse the action of the drug was found to be one molecule to 25. Consequently, on adaptation to sulphathiazole, this ratio would have to be reached by the P.A.B.A., if we are to accept an increased production of this substance as the mechanism of drug adaptation.

While the molar ratio of total diazotizable amine to sulphathiazole occasionally exceeded this and was in fact in one case as high as 1 : 3, the P.A.B.A./S.T. ratios were much lower, of the order 1 : 500, and never approached the required figure of 1 : 25. It is therefore clear that adaptation of *Bact. coli* to sulphathiazole cannot be explained on the basis of increased production of P.A.B.A.

Likewise, our results do not give any indication that anthranilic acid plays any rôle in this connection. The ratio of anthranilic acid to sulphathiazole required to counteract the latter is much higher than that of P.A.B.A. to sulphathiazole and is of the order of one molecule to one. The quantities produced do not come anywhere near this requirement.

(5) While the aromatic amino compounds described above were obtained by bacteria in the absence of sulphonamides, or by sulphonamide-adapted bacteria, a different diazotizable amine has been isolated by Stetten and Fox (1942, 1945) by unadapted organisms growing in the presence of sub-bacteriostatic concentrations of sulphonamide; its amount exceeded that of our substances a hundredfold. Stetten and Fox did not indicate whether they could obtain the substance from adapted organisms, but stated that, when sulphonamide inhibition was reversed by addition of P.A.B.A., the substance no longer appeared. An attempt on our part to isolate this amine from a culture of adapted organisms growing in the presence of sub-bacteriostatic concentrations of sulphathiazole was unsuccessful.

We are forced to the conclusion that p-aminobenzoic acid production cannot be invoked to explain the phenomenon of acquired drug resistance, so far as *Bact. coli* is concerned. Earlier workers found increased production by resistant organisms of sulphonamide antagonists, the presence of which was determined biologically, and which appeared to be identical with p-aminobenzoic acid. It may be that the mechanism of drug-adaptation is different in different organisms and that staphylococci, for example, do produce more p-aminobenzoic acid, although this again has not been confirmed (cf. Liebermeister, 1943). In *Bact. coli*, at any rate insufficient p-aminobenzoic acid to explain the drug-resistance was found in the supernatant fluid. While it is difficult to disprove the assumption that the concentration of P.A.B.A. inside the resistant organisms may be much higher than that in the culture medium, we have found no experimental support for it. On the contrary, we have failed to find P.A.B.A. in the cells even when it was found in the supernatant.

If we are to retain the Woods-Fildes theory that an enzyme operating on p-aminobenzoic acid as substrate is inhibited competitively by sulphonamides, then we must interpret drug-resistance as demonstrating the development by the

organism of an alternative metabolic mechanism to that inhibited. While there is no evidence that the mechanism is the same for all organisms, the failure to find an increase in p-aminobenzoic acid production by one organism necessitates full investigation of others, using equally specific methods.

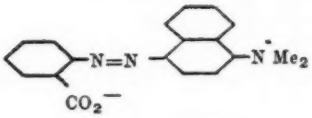
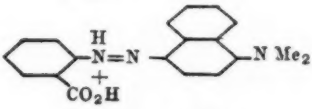
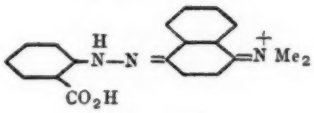
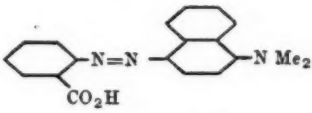
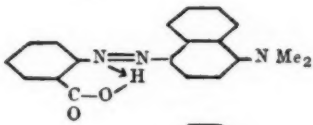
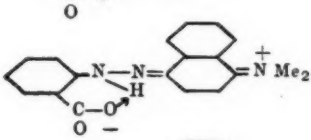
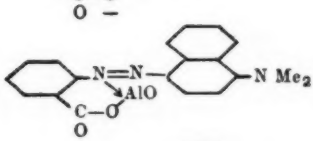
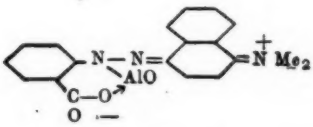
| | Charge. | Colour. | Dye from o-aminobenzoic acid. | Dye from p- and m-aminobenzoic acids. |
|---|---|---|---|---|
| I | anion | yellow |  | same |
| II, a | kation | purple |  | same |
| II, b | | |  | |
| III | neutral | orange |  | same |
| IV, a | neutral dipole (in water, solid) | blue-violet |  | not existing |
| IV, b | | |  | |
| V, a | alumina-adsorbed | blue-violet |  | not existing |
| V, b | | |  | |

**Fig. 1.** Characteristic differences between azodyes of aminobenzoic acids.

The nature of the second dye obtained from anthranilic acid is not yet certain. Since it can be partly converted into the first dye, it may be a cis-trans isomeride.

From our work it is also apparent that the use of the ordinary Bratton-Marshall method of sulphonamide estimation for the determination of p-aminobenzoic acid in biological fluids can lead to serious errors. Anthranilic acid and other diazotizable amines may accompany p-aminobenzoic acid, and the concentration of the latter may be very small as compared with that of the other amines. Previous work with urine has shown us that aromatic amino compounds, other than p-aminobenzoic acid, also occur in normal urine.

*Note on the Chemical Structure of the Azo-Dyes.*

It has been shown that the dye obtained by coupling diazotized anthranilic acid with dimethyl-α-naphthylamine differs characteristically from the corresponding dyes obtained from the p- and m-compounds. Whereas solutions of the three dyes in hydrochloric acid or sodium carbonate do not differ in colour and absorption spectrum, and the ether solutions are also of rather similar colour, the solid dye from anthranilic acid, its adsorbate to alumina from ether-acetic acid, and its solutions in water or dilute acetic acid are of violet colour resembling that of the hydrochlorides. No such forms are observed with the dyes from p- and m-aminobenzoic acids. This difference can be explained in the way illustrated in Fig. 1. Only in the dye produced from anthranilic acid is the hydrogen of the carboxyl group in a steric position, allowing it to chelate by hydrogen bondage with one of the nitrogen atoms of the azo groups. This evidently stabilizes the dipole resonance form IVb, with separated charges in its quinoid structure of the naphthalene nucleus. This resembles the kationic form IIB[4]. It is of interest that the difference of the corresponding dyes produced from the amino acids by diazotization and coupling with dimethylaniline is far less marked. In the alumina adsorbates the anthranilic acid dye probably forms an aluminium-chelated ring with similar resonance form Vb.

## SUMMARY.

A method for the separation and identification of small amounts of p- and o-aminobenzoic acids has been developed. The acids are diazotized and coupled with dimethyl-α-naphthylamine and the azo dyes so formed are separated by chromatography.

A strain of *Bact. coli commune*, growing in synthetic Kisch's medium, produced diazotizable amino acids in the supernatant. The major part of these could be shown to consist of anthranilic acid with only a small amount of p-aminobenzoic acid.

After this strain had become adapted to sulphathiazole, it did not produce more p-aminobenzoic acid than the parent susceptible strain, and the amount of

[4] While the latter form is present in the kations of all dyes, the neutral dipole form IVb is only present in the anthranilic acid dye.

p-aminobenzoic acid was insufficient to account for the resistance to sulphathiazole. The same held for the sum of o- and p-aminobenzoic acid produced.

The production of anthranilic acid was variable and decreased with the number of sub-cultures.

The characteristic differences between the dye derived from o-aminobenzoic acid and those derived from p- and m-aminobenzoic acids are explained as due to the chelation of the o-carboxylic acid group with the azo group nitrogen by aluminium or hydrogen linkage.

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# DESTRUCTION OF INFLUENZA VIRUS RECEPTORS IN THE MOUSE LUNG BY AN ENZYME FROM *V. CHOLERAE*

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Recent investigations of Burnet and colleagues have dealt with an enzyme-like component of the filtrates of *V. cholerae* cultures. Conditions for production and optimal activity have been published (Burnet, McCrea and Stone, 1946; Burnet and Stone, 1947), and the effect of the filtrates on various biological substrates studied. The most extensive studies have been concerned with the power of such filtrates to remove from erythrocytes the "receptors" involved in haemagglutination by the viruses of the mumps-influenza group. Quantitative work has shown that the filtrates can repeatedly induce these changes with very little loss of activity. Taken along with other evidence, this allows the assumption that the active agent is an enzyme, which in this laboratory is referred to as the receptor destroying enzyme (RDE) of *V. cholerae* (Stone, 1947a, b).

The chief interest of the enzyme lies in its relation to virus action. Burnet has suggested that the initial action of mumps-influenza viruses on the cell is mediated by an enzyme very similar to RDE. The similarity of action is increased by the observation that the nonspecific inhibitor of virus haemagglutination in normal sera described by Francis (1947) is destroyed by the enzyme (Anderson and McCrea, 1948), and by Stone's experiments which show that the propagation of living influenza virus is also interfered with by these filtrates (Stone, 1948).

The present paper reports a study of RDE action on cells known to be susceptible to infection by influenza virus, viz. those lining the lower respiratory tract of the mouse. The experimental methods are similar to those used by Hirst (1943), though the original technique had to be modified in certain details to allow reproducible quantitative results.

## MATERIALS AND METHODS.

*Virus Strain.*

The LEE strain of influenza B grown in fertile hen's eggs was used throughout the experiments. The harvested allantoic fluids were tested for their agglutinin titres and freedom from bacterial contamination. Those not contaminated and of higher titres than 200 were pooled and stored at 4° C.; they are referred to in this paper as "active". "Heated virus" was prepared by heating allantoic fluids for 30 minutes in a water-bath at 56° C. No allantoic fluid older than 12 days was used.

*"Receptor Destroying Enzyme" (RDE).*

Plates of 1 p.c. agar were inoculated with a few drops of broth cultures of the "4Z" strain of *V. cholerae* and incubated for 16 hours at 37° C. The surface growth was scraped off, the agar removed into a gauze bag, and the fluid pressed out through a Büchner funnel. The fluid was centrifuged to remove most bacteria, and passed through a Seitz filter. Filtrates were heated in the presence of excess calcium ions at 55° C. for 30 minutes. The fluid so obtained had a yellowish colour, was completely transparent, of a pH between 5·9 and 6·5. Its titre varied from 1,000 to 2,000. These filtrates were stored at 4° C, and in the experiments were used as dilutions giving titres between 350 and 400.

(For further details of RDE preparation see Burnet, McCrea and Stone, 1946; Burnet and Stone, 1947.)

*Miscellaneous Preparations.*

*Normal Saline.* 8·50 gm. NaCl in 1,000·0 gm. distilled water.

*Citrate Saline.* 8·50 gm. NaCl and 10·00 gm. Na citrate per 1,000·0 gm. distilled water.

*Calcium Saline.* 1·00 gm. $CaCl_2$, 8·50 gm. NaCl, 1·203 gm. $H_3BO_3$, 0·052 gm. $Na_2B_4O_7 \cdot 10 H_2O$ in 1,000·0 gm. distilled water. The pH lay between 7·1 and 7·3.

*Red Cells.* Blood was obtained from the wing vein of adult fowls, the cells washed three times with normal saline and made up to a 2 p.c. suspension. Between experiments cells were kept in the refrigerator and no cells older than 36 hours were used.

*Preparation of Mouse Lungs.*

Six to seven-weeks-old laboratory mice of the same breed were used in the experiments. They were killed by destroying their medulla oblongata under light ether-chloroform anaesthesia by pithing. The axillary vessels on both sides as well as the abdominal aorta and vena cava were cut before opening the thoracic cavity. After piercing the diaphragm and causing the collapse of the lungs by this means, the costo-cartilaginous line was cut through with scissors and the chest cavity widely opened by removing the major part of its anterior wall. The heart was excised *in situ*, and the trachea-lung complex dissected out beginning from immediately below the larynx. The outer surface of the lungs was rinsed by dipping into normal saline, and the upper end of the trachea slipped over a glass cannula inserted in a one-hole rubber stopper, and tied to it by thread. The stopper then was placed in a glass vessel which was essentially a short test tube with a side arm by which the air pressure in the vessel could be increased or decreased. It contained sufficient liquid paraffin to provide support for the lungs, the level being adjusted to reach the hilus of the suspended lungs. It was found desirable that a bubble of air should remain under the diaphragmatic surface of the lungs so as to release the unphysiological strain exerted on the trachea and the bronchial tree by suspending the lungs from them. Damage to the lungs either by the instruments used or by incautious handling was carefully avoided. With this method it was possible to avoid almost completely the considerable leakage of fluid which is observed in prolonged experiments of this type when the lungs are suspended in air. To the side arm of the flask a rubber tube was attached, and by appropriate blowing and sucking the suspended lung could be caused to contract and expand.

The fluids with which the respiratory tract was treated were measured into the glass tube from which the lungs were suspended by a calibrated capillary pipette, and aspirated by gentle suction. After a certain time the pressure in the container was raised, the fluid came up into the tube, and the quantity regained was removed and measured with the same pipette with an accuracy of $\pm 0{\cdot}02$ ml. (4 p.c.). If samples had to stay in the lungs for more than one minute, by alternate pressure and suction the fluid was brought up into the glass tube and mixed by

this procedure approximately every minute. The average time needed to aspirate and expel 0·50 ml. fluid to and from the lungs was 5–10 seconds.

Previous to experiments all lungs were washed with three changes of 0·50 ml. normal saline; lungs whose washing fluids contained blood were discarded. The quantity of fluid which could not be pressed out is referred to as "residual quantity", and serves as a basis for correction of results.

*Titration of Virus Haemagglutinin.*

The routine technique used in this Institute for estimating haemagglutinin titres had to be slightly modified, as the samples containing virus contained in most cases RDE too. As the activity of RDE on red cells is greatly reduced in the absence of Ca ions, all dilutions were made in citrate saline. Experiments showed that 10 agglutinating doses of virus could be determined without difficulty in media containing 1,000 inhibiting doses of RDE. Virus suspensions agglutinated to the same titres in citrate saline as in normal saline.

0·25 ml. of 2 p.c. red cells was added to 0·25 ml. citrate saline containing doubling dilutions of the samples taken. Readings were made an hour later by observing the pattern of the sedimented cells.

*Titration of RDE.*

Doubling dilutions in 0·25 ml. calcium saline were made, and an equal amount of 1 p.c. red cells added. The fluids were mixed thoroughly and placed in a water-bath of 37° C. for 30 minutes. At the end of this time a drop (approximately 0·04 ml.) of active virus suspension containing 20 agglutininating doses was added, the racks shaken and put back for another 30 minutes into the bath. Readings were made by estimating "+" agglutination from the pattern of settled cells. (For full details see Burnet and Stone, 1947).

As virus interfered to a certain degree with the titration of RDE, an empirical Table was prepared from results obtained by titrating standard amounts of RDE in media containing falling dilutions of LEE virus. By this means it was possible to correct RDE-values by looking up in the Tables the apparent RDE-titres and the virus agglutinin titres as determined by the routine technique.

All experiments and the titration of virus agglutinins were made at room temperatures, ranging from 17·5° C. to 22° C.

## EXPERIMENTS.

In the *first series of experiments* — a typical example of which is shown in Table 1 — 0·5 ml. of RDE diluted 1 : 3 in calcium saline was aspirated into the lungs. Controls were washed with the same amount of the diluting fluid alone. One minute later the solutions were removed and replaced by the same amount of calcium saline, which was changed three times at one minute's intervals. In the fifth minute from the beginning of the experiment undiluted allantoic fluid containing active virus was added, left in the lungs for five minutes, removed and replaced by three changes of calcium saline. In the twenty-fifth minute another dose of allantoic fluid, followed by three washings. From this time on fluids were changed every fifteen minutes till the end of the third hour. Finally, 0·5 ml. of RDE : 3 was aspirated, into all lungs, removed after five minutes and followed by three washings at five minutes' intervals.

TABLE 1.

| Time min. | Added ·50 ml. | Removed ml. | Agglutinin titre. | RDE titre. | Added ·50 ml. | Removed ml. | Agglutinin titre. | RDE titre. |
|---|---|---|---|---|---|---|---|---|
| −15 | S | ·40 | | | S | ·40 | | |
| −10 | S | ·45 | | | S | ·50 | | |
| −5 | S | ·50 | | | S | ·50 | | |
| 0 | *RDE* | ·48 | | 200 | S | ·50 | | |
| 1 | S | ·55 | | 140 | S | ·50 | | |
| 2 | S | ·50 | | 30 | S | ·50 | | |
| 3 | S | ·50 | | 8 | S | ·50 | | |
| 5 | *Vir* | ·45 | 240 | – | *Vir* | ·45 | 25 | |
| 10 | S | ·55 | 30 | | S | ·50 | 8 | |
| 15 | S | ·50 | – | | S | ·53 | – | |
| 20 | S | ·50 | – | | S | ·50 | – | |
| 25 | *Vir* | ·48 | 220 | | *Vir* | ·50 | 17 | |
| 30 | S | ·50 | 60 | | S | ·50 | 10 | |
| 35 | S | ·52 | – | | S | ·50 | – | |
| 40 | S | ·50 | – | | S | ·50 | – | |
| 45 | S | ·50 | – | | S | ·50 | 8 | |
| 60 | S | ·50 | – | | S | ·50 | 24 | |
| 75 | S | ·50 | – | | S | ·50 | 60 | |
| 90 | S | ·50 | – | | S | ·50 | 140 | |
| 105 | S | ·50 | – | | S | ·50 | 100 | |
| 120 | S | ·50 | – | | S | ·50 | 40 | |
| 135 | S | ·50 | – | | S | ·50 | 17 | |
| 150 | S | ·50 | – | | S | ·50 | 10 | |
| 165 | S | ·50 | – | | S | ·50 | 8 | |
| 180 | *RDE* | ·50 | – | 320 | *RDE* | ·45 | 80 | 120 |
| 185 | S | ·50 | – | 60 | S | ·55 | 15 | 80 |
| 190 | S | ·50 | – | 20 | S | ·50 | 8 | 35 |
| 195 | S | ·50 | – | – | S | ·50 | – | 17 |
| 200 | S | ·50 | – | – | S | ·50 | – | 8 |
| | Experiment | | | | Control | | | |

*RDE*: Filtrate of *V. cholerae*, titre 380.

*Vir*: Allantoic fluid containing LEE virus, titre 280.

S: Buffered calcium saline.

–: Titre less than 8.

In all samples haemagglutinin titres were determined, and RDE was estimated by the technique described above in the first five washings which followed RDE administration. The results show clearly that while virus is readily adsorbed to the respiratory surface of untreated lungs (less than 10 p.c. free virus in 5 minutes; less than 3 p.c. in 10; less than 1 p.c. in 15), the action of RDE renders the lung incapable of adsorbing virus. With a second dose of virus the phenomenon is repeated: rapid quantitative adsorption to normal lung, no adsorption whatever in RDE treated lungs. After a certain time — in approximately 60 minutes with the quantity of virus used in these experiments — spontaneous elution from the control lungs begins, which has no parallel in the RDE treated

ones. At the end of the experiment a second dose of RDE removes all the virus left in the previously untreated lung, but no virus could be removed from lungs which had been treated with RDE at the beginning of the experiment.

If we repeat the experiment with heated virus, the effect of RDE is shown even more strikingly. There is no adsorption of virus to the lungs previously treated with RDE. The allantoic fluid is merely diluted with the residual quantity of fluid in the lungs, but the total content of agglutinating doses is not diminished thereby. So the picture is exactly the same as with active virus in RDE-treated lungs. In the corresponding control lungs virus is adsorbed quantitatively, seemingly more rapidly than active virus, and the whole of the virus is retained by the lung even after three hours, as the eluting capacity of virus is almost completely destroyed by heating to 56° C. for 30 minutes. Finally, the second dose of RDE does not remove any virus from lungs treated previously with RDE, while it liberates in a relatively short time all of the heated virus adsorbed to the normal lungs.

In a *second series of experiments* virus was adsorbed to the lungs and this treatment followed by the administration of RDE.

Inactive virus is adsorbed quantitatively to the lungs in a short time. It does not come off spontaneously and we did not succeed in removing it by repeated washings of the respiratory surface. But if washings were made with solutions of RDE, the virus was rapidly liberated and it could be shown that the total amount recovered, as judged by the agglutinin content of successive washings, equalled that contained in the allantoic fluid added to the lungs at the beginning of the experiment. This means that heated virus, incapable of spontaneous elution, could be removed quantitatively from the lungs by the action of RDE. In control lungs which were not treated with RDE, there was no liberation of virus even in 48 hours. The administration of RDE to these lungs removed 70–90 p.c. of the virus originally added, the liberation being less than quantitative from the eighth hour of the experiment onwards.

The adsorption of active virus to the lungs is practically complete in 15 to 20 minutes, and after a certain interval during which washing fluids do not contain measurable quantities of virus, spontaneous elution begins. Under the conditions of these experiments this commenced between the fiftieth and fifty-fifth minute from the administration of the virus. From this time on all washing fluids contain virus, till the quantity regained reached about 65–85 p.c. of the quantity originally added. This limit once reached — approximately at the end of the third hour — there is no further elution even in 48 hours.

There are three phases of the process of adsorption and elution of virus at which experimental interference with RDE is possible: (1) in the period of latency, when adsorption is complete and elution not yet begun; (2) during elution; and (3) after spontaneous liberation of virus has ceased. A graphical representation of results of these three types of experiment is to be seen in Fig. 1.

RDE added to the lungs during the latent period following the adsorption of active virus removes the virus at a rate much higher than the rate of spontaneous elution. This liberation starts immediately after RDE reached the alveoli, irrespective of the time at which spontaneous elution is due to begin. The quantity of virus originally adsorbed was regained quantitatively from the washing fluids after RDE treatment. Compared with this total removal of virus, there is no detectable amount of agglutinin in the washings of the control lungs during the same period of time.

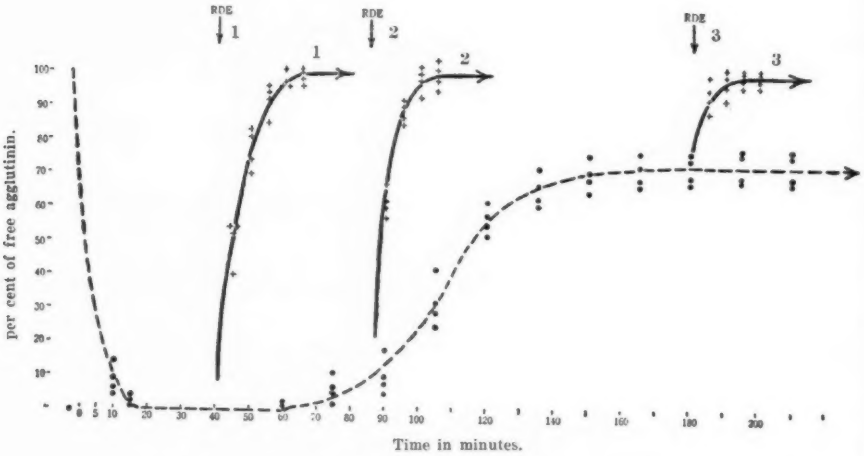


**Fig. 1.** The action of RDE in liberating virus from the excised mouse lung.
- - - Normal adsorption-elution curve of virus in untreated lungs.
— Artificial liberation of virus by RDE.

If RDE is administered while elution is in progress, virus is liberated at a far higher rate from the respiratory surface than in the controls. This difference is to be seen clearly on the graph. The curve of artificial liberation is steep, and reaches its maximum — which is at the same time the total quantity of virus adsorbed — in a very short time. The curve of spontaneous elution converges to an asymptote at the level of about 70–80 p.c. of virus added, and reaches this lower maximum in about 50 times the time needed for artificial liberation to be complete.

In the last series RDE was added after elution of active virus had ceased. It was found that the residual quantity of virus which did not elute spontaneously was removed by RDE. The intervals between the administration of allantoic fluid and that of RDE ranged from 3 to 48 hours. In the first ten hours the removal of the residual quantity of virus was achieved in a few minutes, the amount of agglutinating doses added at the beginning of the experiment being

regained without significant loss. From this time onward liberation of virus by RDE became more or less incomplete. This is probably to be related more to the occurrence of histolytic changes in the lungs than to any changes in the adsorption of the virus.

## DISCUSSION.

These experiments were initiated to determine whether the action of RDE on cells susceptible to virus infection was essentially similar to its action on red cells. The results show clearly that culture filtrates from *V. cholerae* containing RDE do in fact render the respiratory surface of excised mouse lungs incapable of adsorbing the LEE strain of influenza virus B. The effect can be shown equally clearly either by the failure of pre-treated lung to retain virus or by the rapid liberation of adsorbed virus—either active or heated—by subsequent treatment with active filtrate.

Three points call for some discussion.

(1) The tacit assumption that the phenomena described are due to the same agent (RDE) that destroys the virus receptors of red cells requires formal justification. Pure RDE is not yet available, but the two standard preparations used in this laboratory are associated with quite different impurities. These are (a) filtrates heated in the presence of excess Ca ions as used in the present study, and (b) eluates from washed red cells on which the enzyme has been adsorbed in the cold.

Eluates of RDE were comparable to the filtrates used above, and produced exactly similar effects. Further, the effect of RDE can be destroyed by treating an active filtrate with an anti-RDE rabbit serum and not with an anti-vibrio serum not containing anti-RDE. These experiments will form the subject of a further communication. They are mentioned only to establish that RDE is the only agent essentially concerned in the present experiments.

(2) Since Hirst (1943) has stressed that perfusion of the lungs is a necessary preliminary to experiments of the present type, some justification is required for abandoning this procedure. The main reason was to reduce the time and labour involved in preparing the lungs and so to allow more extensive experiments. The change was not made, however, until a careful comparison between adequately perfused and unperfused lungs had been completed. In addition to showing that there was no demonstrable difference in the rate of adsorption and elution of virus or in the activity of RDE, the following additional results were obtained:

a. The first washings from excised lungs, whether perfused or unperfused, contain the same quantity of "Francis' inhibitor" (Francis, 1947) acting on heated LEE virus, provided there is no visible haemoglobin.

b. When inhibitor is present in larger quantities its amount is proportional to the amount of haemoglobin in the sample.

c. Small amounts of blood (0·05 ml. 2 p.c. RBC suspension) do not interfere significantly with the adsorption and elution of virus either in perfused or in non-perfused lungs.

It appears that with careful handling of normal lungs there is little likelihood of blood getting into the bronchial system. Even if a little blood should be present, the extra virus receptors so introduced are a negligible quantity in comparison with the receptors of the respiratory surface.

(3) All experiments have been done in quadruplicate and repeated at least twice. An analysis of the results plus those of further experiments specially designed to determine the accuracy of the procedures showed:

a. Between the adsorption-elution curves of different lungs given the same amount of virus the differences are less than 25 p.c. The total amount of virus recovered from different lungs, as judged by the sum of agglutinating doses, varied by less than 25 p.c. In other words, the reproducibility of the results was limited only by the accuracy of the haemagglutinin titrations.

b. The loss of fluids during the experiments, due to leakage from the lungs, was less than 5 p.c.

Within the limits imposed by the artificiality of the conditions it can be claimed that the methods described in this paper provide a simple and accurate means of investigating the kinetics of influenza virus adsorption by cells naturally susceptible to infection with the virus.

## SUMMARY.

By treatment with preparations from filtrates of *V. cholerae* containing the enzyme RDE, excised mouse lungs are rendered incapable of adsorbing influenza virus B (Strain LEE).

Such preparations remove adsorbed heated or active virus quantitatively from the lung.

The results are consistent with the hypothesis that the *V. cholerae* enzyme destroys the receptors by which influenza viruses make their initial effective contact with susceptible cells.

*Acknowledgment.* The work reported in this paper was begun at the suggestion of Professor F. M. Burnet to whom I am indebted for interest and criticism during its course.

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# THE INFLUENCE OF HEPARIN ON THE TOXICITY OF AUSTRALIAN SNAKE VENOM

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It has been shown by Ahuja, Veeraraghavan and Menon (1946) that heparin significantly reduces the toxicity of the venoms of *Echis carinatus* and the Russell viper, which are known to have a marked coagulant effect. Since some of the Australian venoms also have thrombotic activity, two venoms from *Pseudechis porphyriacus* and *Notechis scutatus* were investigated and an attempt made to determine the effect of heparin on their toxicity.

## METHOD.

The venoms of the Australian tiger snake[2] (*Notechis scutatus*) and of the black snake (*Pseudechis porphyriacus*) were used. Tiger snake venom was injected subcutaneously into a series of guinea-pigs and the $l.d._{50}$ determined. Using almost double this dose, a series of guinea-pigs was injected with venom and immediately after with heparin and daily thereafter. A control series of pigs was injected with venom alone. The animals were observed closely and the results tabulated, the death times being taken. With black snake venom mice were used and the same procedure observed, the timing of the deaths being recorded more accurately, this being advisable and possible owing to the quicker deaths with black snake than with tiger venom.

## EXPERIMENTAL.

*Notechis scutatus* (Australian tiger snake).

A series of guinea-pigs was injected subcutaneously with decreasing doses of venom from 2·33 mg./kg. down to 0·0046 mg./kg. It was found that survivals were only present where animals received less than 0·0065 mg./kg. venom (Table 1). Using the last groups in Table 1, the $l.d._{50}$ was determined by the method of probits. This was found to be 6·7γ/kg. Two groups of eight animals were injected with 0·016 and 0·0133 mg. of venom/kg. and half the animals in each group received 1 mg. of heparin/kg. daily. None of the animals survived. It was, therefore, considered unlikely that heparin had any marked effect in reducing mortality due to this venom.

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[1] This work was aided by a grant from the National Health and Medical Research Council.

[2] Thanks are due to Professor Burnet, F.R.S., Director of the Walter and Eliza Hall Institute, for the supply of tiger snake venom.

TABLE 1.

*Effect of tiger snake venom* (Notechis scutatus) *on guinea-pigs.*

| No. of pigs. | Dose (mg./kg.). | Effect. | Death time (days). |
|---|---|---|---|
| 1 | 2·33 | + | less than 1 hour |
| 1 | 0·89 | + | less than 1 hour |
| 1 | 0·51 | + | less than 1 day |
| 1 | 0·30 | + | less than 1 day |
| 2 | 0·15 | ++ | both less than 1 day |
| 2 | 0·10 | ++ | both less than 1 day |
| 2 | 0·067 | ++ | both less than 1 day |
| 2 | 0·044 | ++ | both less than 1 day |
| 2 | 0·031 | ++ | both less than 1 day |
| 5 | 0·022 | +++++ | 1, 1, 2, 3, 4, days |
| 4 | 0·0188 | ++++ | 2, 2, 2, 3 days |
| 5 | 0·016 | +++++ | all 3 days |
| 4 | 0·0133 | ++++ | all 2 days |
| 4 | 0·011 | ++++ | 1, 3, 4, 7 days |
| 4 | 0·0091 | ++++ | 3, 3, 3, 7 days |
| 8 | 0·0065 | ++++SSSS | 3, 6, 7, 8 days |
| 4 | 0·0046 | SSSS | — |

+ = death. S = survival.

Then six animals were injected with 0·011 mg./kg. of venom, two receiving venom alone and four receiving 1 mg./kg. of heparin in addition (Table 2). The injections of heparin were continued daily thereafter. All animals died. The non-heparinized animals (2) died in 35 and 60 hours. The treated animals died in 41, 41, 112 and 156 hours. It appeared from these groups that the toxicity of venom was not decreased by heparin but it was thought worth-while to investigate if the death time was prolonged following treatment with heparin.

TABLE 2.

*Effect of heparin on envenomed (*Notechis scutatus*) guinea-pigs.*

| Venom alone. | | Heparin + venom. | |
|---|---|---|---|
| No. of pig (in order of death). | Death time (hours). | No. of pig (in order of death). | Death time (hours). |
| 1 | 35 | 1 | 41 |
| 2 | 60 | 2 | 41 |
| | | 3 | 112 |
| | | 4 | 156 |

Eighteen pigs were randomized into two groups, each of nine. The first received 11γ/kg. of tiger snake venom but no heparin, whilst the second group received 11γ/kg. of venom and immediately following that a second injection of 1 mg./kg. of heparin. The animals which received heparin were given daily injections of this material thereafter. The animals were set aside and their death times recorded (Table 3). In each group one pig survived.

TABLE 3.

*Effect of heparin on envenomed* (Notechis scutatus) *guinea-pigs.*

| Venom alone. | | Heparin + venom. | |
|---|---|---|---|
| No. of pig (in order of death). | Death time (hours). | No. of pig (in order of death). | Death time (hours). |
| 1 | 22 | 1 | 34 |
| 2 | 34 | 2 | 48 |
| 3 | 34 | 3 | 56 |
| 4 | 48 | 4 | 84 |
| 5 | 58 | 5 | 108 |
| 6 | 58 | 6 | 130 |
| 7 | 67 | 7 | 153 |
| 8 | 108 | 8 | 201 |
| 9 | S | 9 | S |

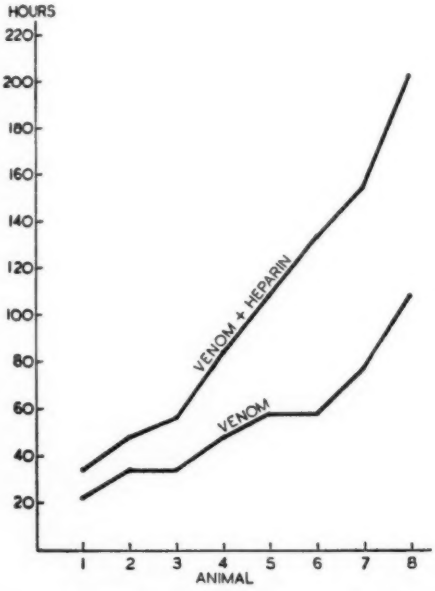


**Fig. 1.** Comparison of death time of guinea-pigs with and without heparin treatment following injection of tiger snake venom. Ordinate: Death time in hours. Abscissa: Number of animals according to death. Details in text.

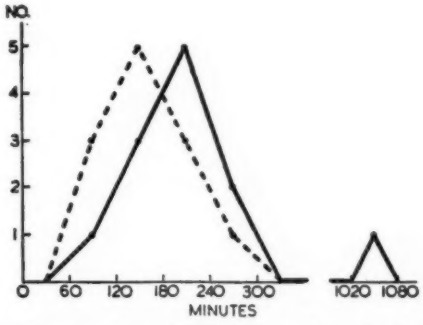


**Fig. 2.** Comparison of death time of mice with and without heparin treatment following injection of black snake venom. Ordinate: Number of mice dying in 60-minute groups (0–60, 60–120, 120–180, 180–240, 240–300, and 1,020–1,080 min.). Abscissa: Death time in minutes. Details in text.

It is noted (Table 3) that of the animals which died and received venom alone, the death time varied from 22 to 108 hours. In the case of animals treated with heparin, the death time varied from 34 to 201 hours. Thus, while there is no reduction in mortality, there was a prolongation of the death time. This effect is set out graphically in Fig. 1. Two of the four heparinized pigs (Table 2) were selected at random, the first and the fourth being the ones chosen. These in addition to the two receiving venom alone were taken into consideration with the animals contained in Table 3. This increased the groups to ten each for death times. The standard deviation was 51·9 and the mean difference 48·7 for this series. Therefore, on statistical analysis the increase in death time caused by heparin is significant.

*Pseudechis porphyriacus* (Australian black snake):

A series of mice was injected with doses of venom ranging from 6·4 mg./kg. to 0·025 mg./kg. (Table 4). Using these figures an approximate l.d.$_{50}$ was determined. This was found to be slightly greater than 1·0 mg./kg. Several groups of animals were then treated, some receiving venom alone and others receiving varying amounts of heparin in addition (Table 5).

TABLE 4.

*Effect of black snake venom* (Pseudechis porphyriacus) *on mice.*

| No. in group. | Dose (mg./kg.). | Result. | Death times. |
|---|---|---|---|
| 1 | 6·4 | + | overnight |
| 1 | 3·2 | + | overnight |
| 5 | 1·99 | +++++ | all overnight |
| 5 | 1·66 | ++++S | all overnight |
| 1 | 1·6 | + | overnight |
| 5 | 1·38 | ++SSS | overnight |
| 5 | 1·15 | +++++ | 4 overnight, 1 four days |
| 5 | 0·96 | ++++S | all overnight |
| 6 | 0·8 | ++SSSS | all overnight |
| 5 | 0·66 | SSSSS | — |
| 1 | 0·4 | S | — |
| 1 | 0·2 | S | — |
| 1 | 0·1 | S | — |
| 1 | 0·05 | S | — |
| 1 | 0·025 | S | — |

+ = death. S = survival.

We see from these figures that heparin has no effect on the toxicity or upon the gross death times. Then the heparin dose was increased to 10 times the venom dose in the case of the last series, 13·5 mg./kg. heparin being administered. This apparently had no effect on the death times; however, it was thought advisable to do another series and observe the death times more accurately.

A series of 24 mice, randomized into two groups of 12, were injected subcutaneously with 1·35 mg./kg. venom. One group received 13·5 mg./kg. of

TABLE 5.

*Effect of heparin on envenomed* (Pseudechis porphyriacus) *mice.*

| No. of mice. | Dose. | Result. | Time. |
|---|---|---|---|
| 10 | 1·8 mg./kg. venom | 7 +, 3S | all overnight |
| 10 | 1·8 mg./kg. venom<br>+3·6 mg./kg. heparin | 9 +, 1S | all overnight |
| 10 | 1·8 mg./kg. venom | 10 +, | all overnight |
| 10 | 1·8 mg./kg. venom<br>+12 mg./kg. heparin | 9 +, 1S | all overnight |
| 10 | 1·4 mg./kg. venom | 1 +, 9S | 3 days |
| 10 | 1·4 mg./kg. venom<br>+1·4 mg./kg. heparin | 4 +, 6S | all overnight |
| 12 | 1·35 mg./kg. venom | 6 +, 6S | all overnight |
| 12 | 1·35 mg./kg. venom<br>+13·5 mg./kg. heparin | 5 +, 7S | 4 overnight<br>1 in 21 hours |

+ = death. S = survival.

TABLE 6.

*Effect of heparin on envenomed* (Pseudechis porphyriacus) *mice.*

| Venom. | | Venom + heparin. | |
|---|---|---|---|
| No. of mouse (in order of death). | Death time. | No. of mouse (in order of death). | Death time. |
| 1 | 105 mins. | 1 | 105 mins. |
| 2 | 115 „ | 2 | 130 „ |
| 3 | 115 „ | 2 | 146 „ |
| 4 | 135 „ | 4 | 178 „ |
| 5 | 147 „ | 5 | 200 „ |
| 6 | 154 „ | 6 | 213 „ |
| 7 | 155 „ | 7 | 214 „ |
| 8 | 156 „ | 8 | 216 „ |
| 9 | 210 „ | 9 | 230 „ |
| 10 | 233 „ | 10 | 247 „ |
| 11 | 239 „ | 11 | 250 „ |
| 12 | 252 „ | 12 | 17 hours (overnight) |

heparin immediately after their venom injection. The effect was noted and death times recorded (Table 6). The untreated animals had death times varying from 105 minutes to 252 minutes, while those of the treated mice varied from

105 minutes to 17 hours. These results are plotted in Fig. 2. The continuous line (heparinized animals) gives a peak at 210 minutes which is a shift to the right, compared with the untreated animals (interrupted line) which show a peak at 130 minutes. Thus in this series the animals receiving heparin died on the average at a longer interval after the injection of venom.

## DISCUSSION.

It has been shown (Ahuja, Brooks, Veeraraghavan and Menon, 1946) that heparin reduces the toxicity of Russell viper venom when injected into animals. In this work the effect of heparin on the toxicity of the venoms of *Notechis scutatus* and *Pseudechis porphyriacus* has been investigated.

The sample of tiger snake venom used was apparently extremely potent, less than 10 $\gamma$/kg. being required to kill a guinea-pig. However, the death time was often quite prolonged (up to 7 days) and the signs typically those of a neurotoxic poison. The black snake venom was much less potent, but quicker acting. If this venom is going to cause death, this almost invariably occurs within 24 hours of injection. Heparin does not seem to affect the toxicity of the two venoms considered at all. However, prolongation of death time occurs in animals treated with this substance.

It is known that these venoms have thrombotic activity. According to Rosenfeld and Rubinstein (1941) the coagulant fraction of the *Notechis scutatus* venom is absolutely non-toxic. This links up with the fact that heparin does not reduce the toxicity of the venom but does not explain why it causes lengthening of death times.

In the Australian snake venoms there appear to be three distinct principles, namely, formation of lysolecithin, a thrombotic and a neurotoxic principle. Two alternatives present themselves to explain the mechanism of action of these three substances in animals. The three effects may not act in co-ordination but at separate times, the thrombotic and lysolecithin effects acting first and the other following. When the animal is heparinized the thrombotic effect is presumably inhibited, leaving the slower acting neurotoxic principle to kill. Thus the death time is lengthened. It is possibly because of the strong lysolecithin effect of black snake venom that there was less significance in the prolongation of the death time with heparin. However, macroscopic post-mortem findings and the symptoms of death appeared to be identical in both treated and untreated animals. It is possible that capillary thrombosis might be less frequent with the heparinized animal.

The other alternative is that all three effects act together, thus the inhibition of one effect would leave only the other two to kill, thereby prolonging the death time. However, if animals received a dose of venom only equivalent to the $l.d._{50}$, heparin might be expected to save life if this second alternative were true. Where doses very close to the $l.d._{50}$ of venom were used it was found that heparin

only prolonged life and did not allow survival. Dragstedt, Wells and Rocha e Silva (1942) have brought forward evidence that heparin inhibits the release of histamine by various injurious agents. If this is also true of *Pseudechis porphyriacus* venom it may account either wholly or in part for the effect of heparin but it is still difficult of interpretation. This aspect will be amplified in a further paper concerning black snake venom and histamine.

These findings may have clinical significance in the treatment of snake bite. It may be found that the life of a person suffering from black snake or tiger snake bite may be prolonged by the administration of heparin. During this period other forms of treatment may then be of value which otherwise would have failed at a late stage. This aspect is now being investigated.

## SUMMARY.

The toxicity of tiger snake venom for guinea-pigs and of black snake venom for mice has been determined approximately.

The subcutaneous administration of heparin lengthens the death times of animals injected with tiger snake and black snake venoms. No decrease in toxicity was observed.

The significance of the findings and their relation to snake bite treatment are discussed.

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# THE EFFECT OF THIAMINE AND CALCIUM SUPPLEMENTS ON THE DEVELOPMENT OF SIGNS OF PYRIDOXINE DEFICIENCY

by C. C. KRATZING

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*(Accepted for publication 12th September, 1947.)*

Marion Richards (1945) has presented evidence that rats raised on a generally poor diet, to which excessive amounts of thiamine and calcium have been added, exhibited signs of pyridoxine deficiency. These signs occurred in suckling rats from mothers fed on this diet. The levels of the other members of the vitamin B complex in the diet were low and the experimental results may have been due to a multiple deficiency of the B complex vitamins which was further exaggerated by the additional thiamine. In the light of these results Richards issued a warning against the "present-day tendency to prescribe vitamin $B_1$ somewhat indiscriminately" in human nutrition. We became interested in Richards' work because of the large scale investigation which was in progress at two women's hospitals in Australia (Schofield *et al.*, 1947). In this study the diet of pregnant women was supplemented with thiamine and milk if it was judged to be deficient in these items. If women with a deficient diet were unable to drink milk, they were given the calcium equivalent in calcium lactate. Although it is not justifiable to draw deductions directly applicable to human nutrition from animal experiments, the findings of Richards were considered of sufficient importance to warrant further investigation. Therefore, efforts were made in this laboratory to reproduce Richards' results, using a diet adequate in all members of the vitamin B complex with the exception of pyridoxine which was present in less than normal requirements. Also the amounts of thiamine which were used as supplements were comparable with the levels more likely to be met in human nutritional problems than those fed by Richards.

## EXPERIMENTAL.

The basal diet was casein 18 gm., sucrose 74 gm., salt mixture (McCollum-Davis 185 with small amounts of copper sulphate, manganese sulphate and potassium iodide added) 2 gm., and an oil mixture (peanut oil 50 gm., oleum vitaminatum 40 gm., wheat germ oil 7 gm., 2 methyl 1:4 naphthoquinone 0·1 gm.) 6 gm. In addition the following synthetic vitamins were provided per 100 gm. food: nicotinic acid 2·5 mg., calcium pantothenate 2·0 mg., inositol 8 mg., riboflavin 0·36 mg., choline chloride 100 mg., pyridoxine hydrochloride 0·1 mg. 0·18 mg. thiamine per 100 gm. food was fed animals on the normal thiamine intake and 0·64 mg. on

the high intake. The above diet supplied approximately 120 mg. calcium per 100 gm. and this amount was doubled in the groups receiving extra calcium by the addition of 120 mg. calcium as calcium chloride per 100 gm. food.

Twenty-four weanling female rats were divided into four groups, matched for litter and weight, and were fed the following diets:

Group 1. The diet containing normal thiamine intake.
Group 2. The diet containing high thiamine intake.
Group 3. The diet containing normal thiamine intake and 100 p.c. extra calcium.
Group 4. The diet containing high thiamine intake and 100 p.c. extra calcium.

## RESULTS.

The growth rates of Groups 1, 2, 3 for the first 90 days (shown in Fig. 1) were normal and almost identical; but that of Group 4 was impaired over the latter half of this period. The mean weights for each group over the last four days of the 90 day period were treated statistically by analysis of variance technique.

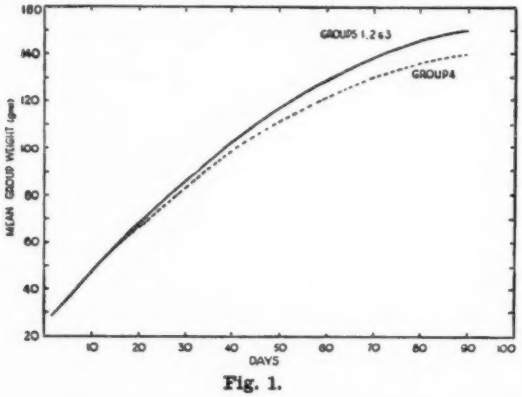


Fig. 1.

The groups varied between themselves significantly at the 5 p.c. level. Comparing individual groups, all differences between Group 4 and the other groups were significant at the 1 p.c. level.

After 90 days the animals were mated with males from the colony and thereafter the resulting young were studied. No fits characteristic of pyridoxine deficiency were seen, although the animals were observed daily during the latter stages of the lactation period. There were losses in young from litters of all groups, but no marked differences could be noted in the percentage of the litters which died from the different groups.

No group differences could be noted in the weights of the weanling rats. The mean weights of the 40-day-old males when compared by analysis of variance method showed no significant group difference at the 5 p.c. level and they were comparable to 40-day-old males raised under colony conditions.

## DISCUSSION.

The results are interesting in that the fits reported by Richards were not noticed. The rats of Group 4 were retarded in weight to a significant level, but their young were not noticeably different from the young of other groups.

Richards' experimental findings may be criticized because the amounts of thiamine used were far in excess of a level likely to be met in human nutrition. Also the quantities of thiamine actually fed were much greater than was apparently intended.

The basal diet used by Richards consisting of flour (61 p.c.), casein (24 p.c.), margarine (10 p.c.) with dried brewer's yeast (1·9 p.c.) and salt mixture (2 p.c.). Three groups of rats were fed this basal diet plus added amounts of thiamine. To the basal diet of Group 1 "aneurin was added to make the $B_1$ content equal to that of the original basal group in the human dietary experiments." Richards stated that this diet contained 198 I.U. per 1,000 Cals. Thiamine was added to the diet of Group 11 (medium aneurin) equivalent to the difference in thiamine content between white and national wheatmeal flour—this diet was reported to contain 554 I.U. per 1,000 Cals. Ten times this difference was added to the diet of "the high aneurin group" (Group III). This contained 3,756 I.U. per 1,000 Cals.

I have recalculated the thiamine content of the diets used by Richards. English, white, unfortified flour and English 100 p.c. extraction flour contain 84 I.U. and 297 I.U. thiamine per 1,000 Cals. respectively (M.R.C. Accessory Food Factors Committee, 1944). It was stated that Group 1 diet contained 198 I.U. thiamine per 1,000 Cals. Of this, 45 I.U. are contributed by the 61 p.c. flour of the diet which supply 53 p.c. of the Calories. If the thiamine content of Group II diet was to contain the difference between white and national wheatmeal flour, the actual content would be 158 I.U. per 1,000 Cals. This means that her "medium" group would have contained less thiamine than the "low" group and in contrast with the 554 I.U. thiamine per 1,000 Cals, fed by Richards.

Similarly, the "high" groups should become 1,175 I.U. per 1,000 Cals. when ten times the difference of the flour levels are taken. This figure is less than a third of that actually fed (3,756 I.U. per 1,000 Cals.).

The feeding of large amounts of thiamine and calcium has the effect of either reducing in some way the amount of pyridoxine obtained by the rat or of increasing its pyridoxine requirements. However, excessive amounts of thiamine in the diet of rats result in a marked increase in urinary excretion of the vitamin, for the animal is unable to store it beyond a saturation level. In addition, the thiamine content of rat milk is unable to be raised beyond limiting levels (Houston and Kon, 1939), so that the young of the "high" thiamine groups received no significantly greater amounts of thiamine than those of other groups. The diets of Richards' Group 1 and Group 1 of this experiment both contained amounts of thiamine which would allow normal growth (Arnold and Elvehjem, 1938), but they were not high enough for complete tissue saturation (Leong, 1937). They are not diets "low" in thiamine, and it is unlikely that further thiamine would cause an imbalance in the metabolism of the vitamins.

It is suggested that the presence of relatively large amounts of thiamine or thiamine and chalk in a flour diet results either in a change in the intestinal flora to a type only capable of limited synthesis of pyridoxine, in a site available to the animal, or to a type having great demands for pyridoxine for their own growth in the way that live yeast removes thiamine from the intestine and so makes it unavailable to the animal (Parsons *et al.*, 1945). The hypothesis is put forward that under Richards' experimental conditions of feeding flour as the carbohydrate,

the addition of excessive thiamine or calcium has changed the composition of the flora in a way which lessens the animal's already low pyridoxine supply. Sucrose does not support the same type of intestinal flora as the more complex carbohydrate, starch (Najjar and Barrett, 1945), so that when sucrose is used these changes do not develop to nearly the same extent. The slow growth rate of Group 4 may have been due to some change in the intestinal flora. Such a hypothesis would explain the discrepancy between the results of the two experiments.

At the present time, the significance of the findings to human nutrition is questioned until it is found whether changes in intestinal flora cause these results and whether human flora would behave similarly.

## SUMMARY.

Female rats on a diet adequate, except for pyridoxine, did not give rise to pyridoxine deficiency in their young, when extra thiamine or extra thiamine and calcium was included in the diet. A hypothesis correlating this result to the findings of Richards is advanced.

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# PREVENTION OF VIRUS INFECTION WITH ENZYME OF *V. CHOLERAE*

## I. STUDIES WITH VIRUSES OF MUMPS–INFLUENZA GROUP IN CHICK EMBRYOS.

by JOYCE D. STONE[1]

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*(Accepted for publication, 9th October, 1947.)*

Following his discovery of the phenomenon of haemagglutination by influenza virus (Hirst, 1941, 1942), Hirst described a somewhat similar reaction between this virus and the cells of the excised ferret lung (Hirst, 1943). In each instance virus was adsorbed on to the susceptible cell and after a short time spontaneously released. Release of the virus was accompanied by modification of the surface of the cell so that it no longer possessed the capacity to adsorb virus and in the case of the red cell to be agglutinated by it.

The recent discovery (Burnet, McCrea and Stone, 1946) that certain bacterial enzymes could destroy the capacity of red cells to adsorb or agglutinate with viruses of the mumps-influenza group immediately raised the possibility of modifying the interaction between virus and susceptible tissue with enzymes of this type. The enzyme produced by *V. cholerae* was chosen for this investigation as it could be readily produced in relatively large amount (Burnet and Stone, 1947) and an extensive study of other aspects of its activity was being made concomitantly in this laboratory. Evidence has recently been obtained (Burnet, McCrea and Anderson, 1947) that the substrate of this enzyme is a form of mucin. For the purposes of this communication, however, the enzyme will be referred to as receptor-destroying enzyme (RDE), a name derived from its action on the virus receptors of the red cell.

Experiments to be reported in this paper have established firstly that RDE can modify the cells of the allantois of the developing chick embryo so that they are no longer capable of adsorbing influenza virus haemagglutinin. In the second place, it was found that allantoic injection of RDE prevented infection of the embryos with viruses of the mumps-influenza group. The extent of this effect varied with the strain of virus. Preliminary results of this investigation have already been reported (Stone, 1947a).

[1] This work was carried out under a grant from the National Health and Medical Research Council, Canberra.

## MATERIALS AND METHODS.

The following strains of virus were used:

Mumps—Enders strain supplied through the courtesy of Miss P. E. Lind.
NDV—Newcastle disease virus of fowls, Victorian strain of Albiston and Gorrie.
MEL—Melbourne strain of influenza A isolated in 1935.
WSE—Classical WS strain of influenza A adapted to chorioallantoic passage.
LEE—Standard influenza B strain.
BEL—Influenza A strain isolated during Victorian epidemic 1942.
SW—Shope's strain 15 of swine influenza.
IAN O and D—O and D phases of influenza A strain isolated in Melbourne from sporadic case in February, 1946 (Anderson and Burnet, 1947). Unlike most viruses in the O phase, IAN O grows readily in the allantoic cavity.
CAM (D)—Influenza A strain isolated in April, 1946, during a minor epidemic in Melbourne (Anderson and Burnet, 1947).

Seed virus was prepared as infected allantoic or amniotic fluid, glycerolated to a final concentration of 33 p.c. and stored at —10° C. The same batch of seed virus was used for inoculation in a number of experiments, the titres being checked at intervals by allantoic titration.

Receptor-destroying enzyme (RDE) was prepared from 1 p.c. agar cultures of *V. cholerae* strain 4Z as described previously (Burnet and Stone, 1947). It was used in the form of agar extract heated at 55° C. for 40 minutes in the presence of excess calcium at pH 6·0 or as the eluates prepared from this heated extract by adsorption and elution from human red cells. The heat treatment of the crude agar extract served to kill the vibrios and also to inactivate other more labile enzymes without affecting the titre of RDE.

RDE titrations were made according to the technique already described (Burnet and Stone, 1947). In some experiments it was found convenient to express RDE concentrations in terms of units. The unit of RDE may be defined as the amount present at the endpoint of the standard method of titration or more fully, as the amount which in a volume of 0·25 ml. brings about partial destruction (to the "one plus" level) of the agglutinability for MEL virus (10 standard agglutinating doses) of an equal volume of 1 p.c. fowl red cell suspension in 1 hour at 37° C. Hence a preparation with a titre of 1,600 contains 6,400 units of RDE per ml.

Anti-RDE rabbit serum was supplied through the courtesy of Professor F. M. Burnet. The properties of this serum, which was prepared by subcutaneous inoculation of RDE eluate emulsified in paraffin with a lanoline base preparation, have been studied by Burnet (1948).

In experiments involving exchange of the fluid in the allantoic cavity, the procedure of draining or filling, which was carried out via a small drilled hole with a Pasteur pipette and teat, was facilitated by the presence of a second hole drilled over the air space. In the study of adsorption of virus haemagglutinin in the allantoic cavity of formalinized embryos, the eggs were rotated slowly in a mechanical device at 35° C. throughout the experiment in order to ensure mixing of the fluid in the allantoic cavity. In the longer term experiments on the prevention of infection in living embryos, the movements of the embryo were considered adequate to ensure mixing except in the initial stage following injection of the RDE when the egg was rotated several times by hand.

Although all RDE preparations injected into embryos were ostensibly bacteriologically sterile, penicillin was added in a concentration of 50 to 100 units per egg. It was also added to the fluid used for the final filling of the allantoic cavity as a precautionary measure

against contamination during manipulation in experiments involving washing out of the cavity.

Titrations of virus haemagglutinin in samples taken from the allantoic cavity in the adsorption experiments were carried out at 4° C. in the presence of 1 p.c. sodium citrate (final concentration) in order to prevent destruction of the receptors of the indicator red cells by residual enzyme in samples from embryos treated with RDE.

In testing embryos for evidence of virus infection the criterion adopted was the presence of demonstrable haemagglutinin in 0·25 ml. of allantoic fluid. The general technique was to chill the sample to 4° C. and add 1 drop (0·025 ml.) of 20 p.c. sodium citrate solution and 0·25 ml. of a chilled 1 p.c. suspension of fowl red cells, or guinea-pig cells in the case of IAN O virus. The adequacy of this technique from the viewpoint of inhibiting the action of RDE present in the samples was tested for each virus under study and confirmed for all except NDV. The red cell receptors for this strain are very readily removed so that it was necessary to use 1 p.c. "calgon" (sodium hexametaphosphate) in place of citrate and to make a reading as early as possible after addition of the red cells. The results obtained with NDV by this method were fully confirmed by the occurrence of specific deaths. With samples from older embryos containing precipitated urates the specific nature of any haemagglutination was determined by testing at a 1:10 dilution or with cells rendered resistant to virus agglutination by pretreatment with RDE.

## EXPERIMENTAL.

### *Effect of RDE on Adsorption of Virus Haemagglutinin to Cells of Allantois.*

These experiments, which were based on those of Hirst (1943) on the adsorption and elution of virus in the excised ferret lung, were carried out firstly to establish that the same sequence of adsorption and elution occurred in the allantoic cavity and secondly to test the effect of RDE on this process.

If the lung was studied in the living ferret, Hirst found that there was again rapid adsorption of virus, but no free virus was detectable until 24 hours after inoculation. Similarly, in the present work, it was found that virus was readily adsorbed on the cells of the living allantoic membrane, but there was no demonstrable elution over the 5 hour test period. However, if the membrane was lightly formalinized, adsorption of virus was followed by elution commencing after 1 to 2 hours. Formalin was chosen for treating the membrane, as earlier tests had shown that it did not interfere with the process of adsorption and elution in the case of the red cell. The concentration used, 0·5 p.c. administered in the virus dilution, brought about death of the embryo within two hours, although the local effect on the surface of the cells lining the allantoic cavity would presumably be very rapid.

The behaviour of swine influenza virus haemagglutinin in the allantoic cavity of 12- or 13-day-old chick embryos was studied as follows. The allantoic cavity was drained and refilled with 5 ml. of saline or saline containing RDE. After 30 minutes this fluid was replaced with 5 ml. of 0·5 p.c. formol saline containing 10–30 standard agglutinating doses from a stock of allantoic fluid virus. Samples of fluid withdrawn at intervals over the period 5 minutes to 5 hours were titrated for haemagglutinin content as described above.

In embryos which received saline only before introduction of the virus, the haemagglutinin titre showed a sharp fall during the first half to one hour and then rose again slowly until at 5 hours it reached half or the full original titre (Fig. 1). When the embryo was treated with RDE in the form of 4Z agar extract (5 ml. of a 1 in 10 dilution, 3,200 units of RDE), the haemagglutinin titre remained at a high level over the 5 hour period. There was frequently a small decrease between the titres of the original fluid and the samples removed from the egg, due in part at least to slight dilution with residual fluid in the cavity. 4Z agar extract in which RDE had been previously inactivated by boiling for 10 minutes did not interfere with the normal process of adsorption and elution of virus. Results of embryos treated in this way were given in detail in a recent communication (Stone, 1947a, Fig. 1).

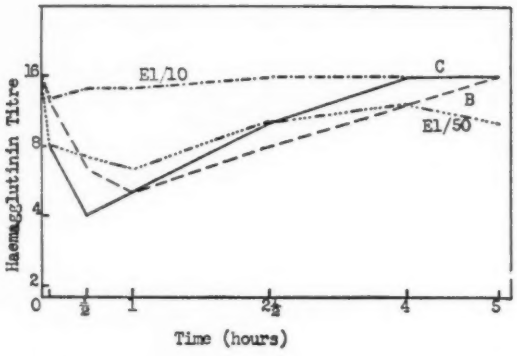



**Fig. 1.** Effect of receptor-destroying enzyme (RDE) on adsorption of swine influenza virus haemagglutinin in allantoic cavity of formalinized embryos. Embryos were pretreated with saline (C), RDE eluate 1/10 (E1/10), RDE eluate 1/50 (E1/50) and boiled RDE eluate 1/10 (B).

Tests carried out with RDE partially purified by elution from red cells gave similar results in that treatment with eluate 1 in 10 (3,200 units) prevented adsorption of virus while eluate previously boiled for 10 minutes and diluted 1 in 10 had no effect (Fig. 1). It will also be seen from Fig 1 that eluate diluted 1 in 50 (640 units) caused no appreciable modification of adsorption.

The fact that this effect in the embryo was obtained with 55° C.-treated agar extract and also with eluates from red cells, strongly suggests that the active agent is RDE. As a further check on the possible activity of components other than RDE in material eluted from red cells, an "eluate" was prepared by the standard technique from 4Z agar extract in which RDE had previously been destroyed by heating at 60° C. for 30 minutes without added calcium. This material failed to prevent adsorption in the embryo. The heat stability of the active agent is therefore in agreement with that of RDE.

A few experiments were also performed in the absence of formalin so that the embryos remained alive throughout. Again, there was no evidence of adsorption of virus in those embryos treated with 4Z agar extract, while the controls showed rapid adsorption but no liberation over the 5-hour period.

*Prevention of Virus Infection in Allantoic Cavity by RDE.*

*Effect of RDE against different strains of virus.* The change induced by RDE in the cells of the allantois rendering them incapable of adsorbing influenza

virus haemagglutinin is accompanied by a decreased susceptibility to infection with viruses of this type. A few preliminary experiments (Stone, 1947a) indicated that infection of the allantoic cavity with small amounts of swine influenza virus could be prevented by allantoic injection of RDE 30 minutes previously. Similar tests have now been carried out with mumps virus, Newcastle disease virus, and the following strains of influenza virus: MEL, BEL, IAN O, CAM, LEE and SW.

A virus dilution, calculated to contain 10–50 $ID_{50}$ for normal embryos was inoculated allantoically into three groups of 10–12-day-old embryos as follows:—

(a) Embryos injected allantoically 30 minutes previously with 0·5 ml. of 4Z agar extract (3,200 units of RDE).

(b) Embryos injected allantoically 30 minutes previously with 0·5 ml. of boiled 4Z agar extract.

(c) Control embryos with no previous treatment.

With mumps virus it was necessary to use a much larger inoculum in order to obtain a high percentage of infections in the control group.

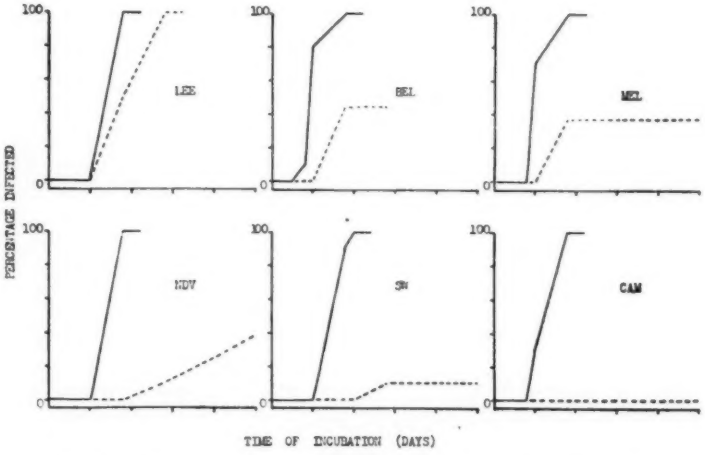



**Fig. 2.** Effect of RDE on allantoic infection with 10–50 $ID_{50}$ of virus. Broken lines represent embryos pretreated with RDE (4Z agar extract, 3,200 units), continuous lines, untreated embryos. Inocula: LEE 30 $ID_{50}$, BEL 10 $ID_{50}$, MEL 50 $ID_{50}$, NDV 50 $ID_{50}$, SW 10 $ID_{50}$, CAM 30 $ID_{50}$. Number of embryos in each group, 8–10.

The results of these experiments are expressed graphically in Figs. 2 and 3, the percentages of surviving embryos with positive haemagglutination being plotted against time of incubation. The number of embryos employed per group is indicated for each strain. With the majority of the strains tested, boiled agar extract had no apparent effect on the development of infection and the

results for these groups have been omitted from the graphs in Fig. 2. With mumps CAM and IAN O, boiled agar extract prevented or delayed infection in a proportion of the embryos but was considerably less effective than the agar extract containing RDE (Fig. 3). The effect of boiled agar extract against these strains can be attributed to constituents present in the original agar medium, as a normal agar extract prepared by the same technique from uninoculated plates had a similar effect on infection with IAN O (Fig. 3b).

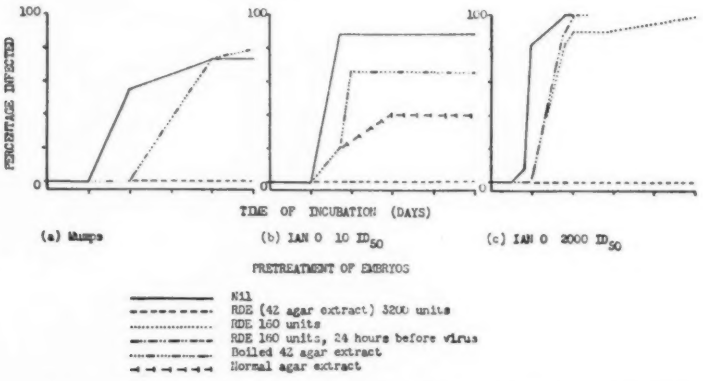



**Fig. 3.** Effect of RDE on allantoic infection with mumps and IAN O viruses.
(a) 9–11 embryos per group. (b) 5–8 embryos per group. (c) 11–12 embryos per group.

With all strains of virus, except LEE, treatment of the embryos with agar extract containing RDE resulted in either complete prevention of infection or prevention in more than 50 p.c. of embryos with a delayed onset of infection in the remainder. In the experiment with LEE virus, shown in Fig. 2, RDE had only a slight retarding effect on infection, although in another test, using a different stock of virus, infection was prevented in four embryos of a group of six receiving RDE.

When embryos receiving a similar amount of 4Z agar extract (0·5 ml.) were inoculated with a larger amount of virus ($10^3$ $ID_{50}$), complete prevention of infection occurred with IAN O (Fig. 3c) and CAM (Fig. 4), but there was no significant effect with MEL or BEL and a partial effect only with SW (Fig. 4).

From these experiments, therefore, there is no evidence that the ease with which virus infection is prevented by RDE corresponds to the sequence in which the different virus receptors on the red cell surface are destroyed (Burnet, McCrea and Stone, 1946). Apart from the greater susceptibility of CAM, IAN O and probably mumps, there appear to be minor strain differences, SW appearing relatively more susceptible and LEE more resistant.

No extensive attempt has been made to study the time factor in the RDE administration. In all the foregoing experiments, RDE was administered 30 minutes before inoculation of the embryo with virus. From the results obtained in the excised mouse lung (Fazekas de St.Groth, 1948) and also in view of the lack of any demonstrable adsorption and elution of RDE in the allantoic cavity, it was considered that the action of RDE on the allantoic cells would take place very rapidly and would be completed in considerably less than 30 minutes. There was certainly no evidence that 160 units of RDE was any more effective against a large inoculum of IAN O (Fig. 3) or a smaller inoculum of MEL (Figs. 5a and 5b) when given 24 hours before the virus instead of 30 minutes before. When RDE (0·5 ml. 4Z agar extract) was administered 30 minutes after inoculation of the embryo with SW virus (10 $ID_{50}$) it delayed infection in approximately half the embryos but had little effect on the number of positive infections after 3 days.

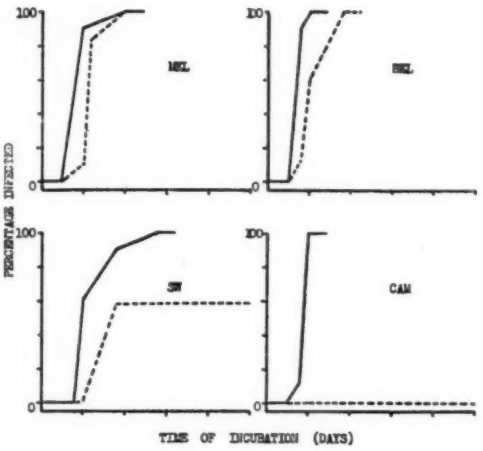



**Fig. 4.** Effect of RDE on allantoic infection with $10^3$ $ID_{50}$ of virus. Broken lines represent embryos pretreated with RDE (4Z agar extract 3,200 units), continuous lines, untreated embryos.

Inocula: MEL 2,500 $ID_{50}$ (12 embryos per group).
BEL 1,000 $ID_{50}$ (8 embryos per group).
SW 1,000 $ID_{50}$ (5–6 embryos per group).
CAM 3,000 $ID_{50}$ (7–10 embryos per group).

Apart from establishing that RDE in the form of red-cell eluates has a similar effect on infection with a typical strain of virus, little work has been done with preparations other than agar extract. The necessity for bacteriologically sterile products in fairly large amount precluded any large scale use of eluates in experiments of this type. In the experiment summarized in Table 1, RDE eluate (3,200 units) delayed the onset of infection with a large inoculum of SW virus (approximately 5,000 $ID_{50}$) while the same volume of boiled eluate and an "eluate" prepared from 4Z agar extract in which RDE had previously been destroyed by heating to 60° C. for 30 minutes were without effect.

To demonstrate that the activity of 4Z agar extract in preventing infection was proportional to the content of RDE, the following experiment was performed. 4Z agar extract from which the bulk of the RDE had been removed by adsorption on red cells was titrated in

parallel with the original agar extract for its activity in preventing infection with a small inoculum of IAN O. The adsorption was carried out according to the standard technique used in the preparation of eluates (Burnet and Stone, 1947) by retaining the first supernatant fluid. The volume of packed human red cells used was 1 ml. per 6 ml. of agar extract.

TABLE 1.

*Effect of RDE eluate on allantoic infection.*

| Pretreatment of embryos.* | Number infected after incubation. | | |
|---|---|---|---|
| | 16 hours. | 24 hours. | 42 hours. |
| Nil. | 0/9 | 9/9 | |
| RDE eluate (3,200 units) | 0/9 | 3/9 | 8/8 |
| Boiled eluate | 0/8 | 8/8 | |
| "Eluate" from 60° C.-treated agar extract | 0/10 | 10/10 | |

* 0·5 ml. of agent indicated was injected 30 minutes before inoculation of SW 1 in $10^4$ (Ca. 5,000 $ID_{50}$).

After adjustment to pH 7·0 the original and absorbed agar extracts were injected allantoically in a series of fivefold dilutions, each embryo receiving 0·5 ml. 30 minutes before inoculation with virus. Although the end points of activity were not clear-cut, it will be seen from Table 2 that the results indicated approximate proportionality between RDE content and activity of the extracts in preventing infection.

TABLE 2.

*Activity of original and absorbed agar extracts in preventing allantoic infection with IAN O (10 $ID_{50}$).*

| Pretreatment of embryos. | Number infected. | | | Pretreatment of embryos. | Number infected. | | |
|---|---|---|---|---|---|---|---|
| | 24 hr. | 48 hr. | 96 hr. | | 24 hr. | 48 hr. | 96 hr. |
| Original extract 1:250 (RDE titre 1,600) | 1/8 | 6/7 | 6/7 | Absorbed extract 1:25 (RDE titre 140) | 0/8 | 5/8 | 6/8 |
| Original extract 1:50 | 0/8 | 3/8 | 4/8 | Absorbed extract 1:5 | 0/8 | 2/8 | 2/8 |
| Original extract 1:10 | 0/8 | 1/8 | 1/8 | Absorbed extract undiluted | 0/7 | 0/7 | 0/7 |
| Nil—controls | 0/8 | 7/8 | 7/8 | | | | |

*Neutralization of RDE with specific antiserum.* Mixtures containing equal volumes of suitable dilutions of rabbit antiserum (R.228) and 4Z agar extract were incubated for 2 hours at 37° C. then injected allantoically, 1 ml. of mixture per embryo, followed after 30 minutes by a dilution of SW virus containing 30 $ID_{50}$. It will be seen from Table 3 that neutralization of the activity of the 4Z agar extract occurred under these conditions with an equal volume of R.228 serum diluted 1 in 10 and partial neutralization with serum 1 in 20. No neutraliza-

tion was obtained with a specimen of normal rabbit serum diluted 1 in 10 while in another experiment not included in the Table, a second specimen of normal rabbit serum failed to neutralize in a dilution of 1 in 2. When the reagents were injected separately into the embryo, 30 minutes before the virus inoculum, the antiserum appeared slightly less effective.

Similar experiments were attempted with IAN O but were inconclusive owing to the inhibition of infection with this strain by normal rabbit sera in high dilution.

TABLE 3.

*Serum neutralization of the action of RDE in preventing allantoic infection with SW virus (30 $ID_{50}$).*

| Pretreatment of embryos. | Number infected after three days. |
|---|---|
| Nil | 8/8 |
| RDE | 1/8 |
| Mixture of RDE + antiserum/10 | 7/7 |
| Mixture of RDE + antiserum/20 | 6/8 |
| Mixture of RDE + antiserum/40 | 0/7 |
| Mixture of RDE + normal serum/10 | 0/8 |
| RDE and antiserum/10 (injected separately) | 5/6 |

*Absence of Direct Effect of RDE on Virus Infectivity.*

The following experimental evidence has been obtained to exclude the possibility that a direct lethal effect of RDE on the virus particle is responsible for the prevention of virus infection described in the foregoing sections. The method depended on the use of a suitable mixture of RDE and virus such that the RDE concentration could be reduced below its effective level by dilution without exceeding the limit of virus infectivity.

TABLE 4.

*Infectivity of SW virus after treatment with RDE.*

| Mixture | Infectivity: number of embryos infected by virus at following dilutions. | | |
|---|---|---|---|
| | $1:10^7$ | $1:10^6$ | $1:10^5$ |
| Virus + 4Z agar extract [1] | 1/6 | 6/6 | 4/4 |
| Virus + boiled 4Z agar extract [1] | 1/6 | 4/6 | 4/4 |
| Virus + 4Z agar extract [2] | 0/5 | 7/9 | |
| Virus + boiled 4Z agar extract [2] | 0/6 | 6/10 | |

Two separate experiments are included.

[1] Virus diluted $1:10^4$ in undiluted agar extract and incubated for 30 minutes at 37° C.

[2] 0·05 ml. of virus dilution 1 in $10^4$, 0·5 ml. of agar extract and 4·5 ml. of normal allantoic fluid incubated for 20 hours at 37° C.

In the first experiment, SW virus was diluted 1 in $10^4$ in 4Z agar extract and in the same material previously boiled for 10 minutes. After incubation for 30 minutes at 37° C., the mixtures were inoculated allantoically in a series of tenfold dilutions. As will be seen from Table 4, there was no significant difference between the infectivity titre of the virus samples exposed to RDE and to boiled agar extract.

In order to simulate more closely the conditions under which virus would be exposed to RDE in the allantoic cavity, a second experiment was performed as follows. 0·05 ml. of swine influenza virus dilution 1 in $10^4$ was added to 5 ml. of normal allantoic fluid containing 0·5 ml. of 4Z agar extract. A second mixture was set up using boiled 4Z agar extract and the two mixtures incubated at 35° C. for 20 hours. The infectivity titre of each was then determined by allantoic inoculation of 0·05 ml. volumes of the mixture undiluted and diluted 1 in 10 (equivalent to dilutions of original virus 1:$10^6$ and 1:$10^7$). Again the titres of the two mixtures were equivalent indicating that RDE had exerted no lethal effect on the virus (Table 4).

The same reagents were used concomitantly to demonstrate prevention of virus infection of the allantoic cavity by RDE according to the technique described earlier. All embryos in the group receiving boiled agar extract showed evidence of infection after incubation for 24 hours, while in the group receiving agar extract containing RDE all embryos were negative after 24 hours, 5 out of 8 becoming positive on the second day.

### *Evidence for Regeneration of Virus Receptors after Destruction by RDE.*

Protection against virus infection by the administration of RDE cannot then be explained on the basis of a direct lethal action on the virus particle but must instead be due to some modifying effect on the interaction of virus with the tissue cell. In view of the associated evidence of the action of RDE on the red cells (Burnet, McCrea and Stone, 1946; Stone, 1947b) and of interference with adsorption of virus to the tissue cell, the effect of RDE on infection may be attributed to a modification of tissue cell "receptors" with which the virus reacts in the initiation of infection.

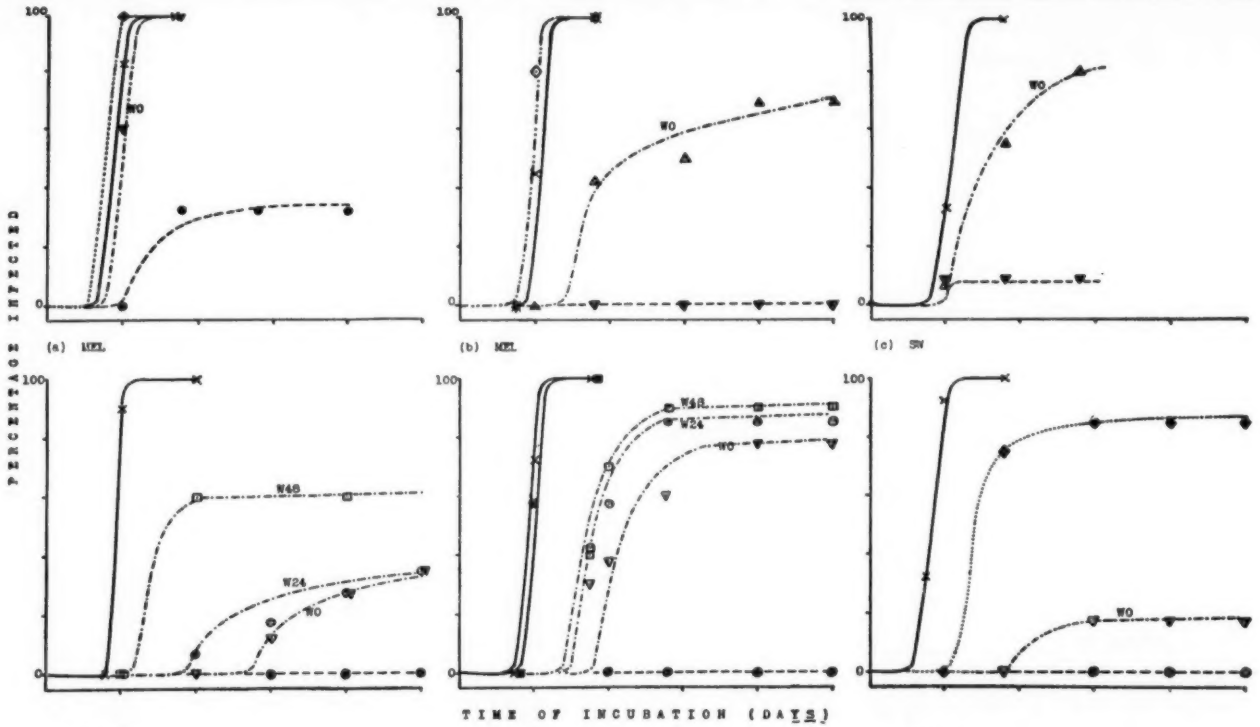
One possibility arising from this hypothesis is that of regeneration of the virus receptors on the surface of the living tissue cell. In the above experiments in which RDE was injected allantoically into the embryo, the concentration of RDE in the allantoic fluid remained practically constant, showing no more than a 50 p.c. drop over the 5-day test period, so that if regeneration did occur it would be immediately counterbalanced by further destruction. Hence, in order to provide opportunity for receptor regeneration to reach a technically demonstrable level, it was necessary, after allowing time for interaction between RDE and tissue cells, to reduce the concentration of RDE below the level which in itself prevented infection. This could be carried out for most strains of virus by simple replace-

EXPLANATION OF FIGURE.

**Fig. 5.** Regeneration after destruction by RDE of virus receptors for:

(a) and (b) MEL 25 $ID_{50}$. (d) and (e) IAN O 2,000 $ID_{50}$.
(c) SW 30 $ID_{50}$. (f) IAN D 2,300 $ID_{50}$.

In groups marked $W_0$, $W_{24}$ and $W_{48}$. embryos were inoculated with virus 0, 24 and 48 hours respectively after washing out of RDE. Calgon saline was used for washing out in (e), in all other cases Ringer solution was used. Groups in (a) and groups marked $W_{48}$ in (d) contained 5–6 embryos, in all other cases groups contained 9–14 embryos.




Pretreatment of embryos.

- Nil.
- Calgon-Saline.
- RDE (3,200 units).
- RDE (160 units).
- RDE (3,200 units), washed out, virus inoculated after 0 hours.
- RDE (3,200 units), washed out, virus inoculated after 24 hours.
- RDE (3,200 units), washed out, virus inoculated after 48 hours.
- RDE (3,200 units), 24 hours before virus.
- RDE (160 units), 24 hours before virus.
- RDE (3,200 units), 24 hours, washed out, virus inoculated immediately.

ment of the fluid in the allantoic cavity. The embryo was then inoculated with virus either immediately or after a specified interval of time.

In the experiments illustrated in Fig. 5a, 0·5 ml. of 4Z agar extract was injected allantoically and after incubation for 30 minutes, the allantoic cavity was drained, washed out and refilled with Ringer solution and inoculated immediately with MEL virus. Using 12- or 13-day-old embryos, it was usually possible to obtain 5–10 ml. of fluid at the first draining and one washing of the cavity with an equal volume of Ringer solution served to reduce the RDE concentration sufficiently. If the fluid obtained at the first draining was less than 5 ml. in volume as was frequently the case with younger embryos, the cavity was washed out twice. This technique enabled a 20–25 fold reduction in the concentration of RDE as determined by titration.

It will be seen from Fig. 5a that, apart from an indication of a slight delay in the onset of infection, these embryos reacted in the same way as the untreated embryos or the embryos receiving 4Z agar extract diluted 1:20. Essentially similar results were obtained in an experiment with SW virus using the same technique.

A possible formal objection to these experiments as a demonstration of receptor regeneration would be that 30 minutes was an inadequate time for RDE to exert its full effect on the cells of the allantois. Tests were therefore made allowing a 24-hour interval in all groups of embryos between the injection of RDE and further manipulation. Using this technique there was definite evidence of some delay in the development of MEL or SW infection in embryos treated with RDE followed by washing out before inoculation of virus (Fig. 5b and 5c). Also, only a proportion of embryos in the group became infected during the period of observation. The levelling off of the graphs for these groups, 2–3 days after inoculation of virus, may well be due, in part at least, to the operation of a secondary factor, namely the dying out of the original inoculum of virus in the allantoic cavity.

In carrying out experiments of this type with IAN O, a large inoculum of virus (ca. 1,000 $ID_{50}$) was used owing to the greater activity of RDE against infection with this strain. As evidenced by Fig. 3, infection with this inoculum was completely prevented with 0·5 ml. of undiluted 4Z agar extract whereas 1/20 of this amount resulted only in a slight delay in the development of infection. Embryos were, therefore, injected with 0·5 ml. of 4Z agar extract and after 30 minutes the allantoic cavity was drained, washed out and refilled with Ringer solution and then inoculated with IAN O (2,000 $ID_{50}$). In embryos treated in this way there was only a small proportion of late infections. When an interval of 24 hours was allowed between washing out the RDE and inoculation of virus the first infections developed somewhat earlier although the percentage of positives after 5 days was approximately the same.

Fig. 5d shows the results of an experiment comprising groups of embryos treated in this way together with control embryos receiving no RDE and embryos in which RDE was allowed to remain throughout the experiment. The manipulations were arranged so that all embryos were inoculated with virus at the same time and were of comparable age. The graph for a group of embryos inoculated

48 hours after washing out of the RDE is included in Fig. 5d for purposes of comparison, although this was obtained on a different occasion and the group comprised only 5 embryos. Taking the results at their face value, however, it seems that the period of time between the washing out of the RDE and the onset of demonstrable infection was approximately constant in all groups. This interval, less the lag between inoculation and onset in normal embryos, may be considered as the time required for regeneration of receptors sufficient to allow growth of this inoculum, at least in a proportion of embryos. A rough estimate of this time may be given as 54 hours.

It was thought that the apparent slowness of regeneration of susceptibility to infection in these experiments might have been due to the continuing low grade activity of residual RDE in the allantoic cavity. As it was technically impracticable to reduce the RDE concentration below this level by simple mechanical removal, attempts were made to inhibit the activity of residual RDE by chemical means using a low concentration of "calgon" (sodium hexametaphosphate). This agent had proved the most powerful of a number previously tested (Burnet and Stone, 1947) for activity in inhibiting the action of RDE on the red cell.

An extensive experiment in which M/1,500 calgon in normal saline was used in place of Ringer solution for washing out the cavity is summarized in Fig. 5e. The general scheme was the same as that of the experiment illustrated in Fig. 5d. An additional control group of embryos was included to determine the effect of calgon-saline on the susceptibility of the embryo to virus infection. It will be seen from the Figure that these embryos, which were drained and refilled with calgon-saline before inoculation with virus, did not differ significantly from untreated embryos in their susceptibility to infection.

In the groups in which RDE administration was followed by washing out with calgon-saline there was a greater proportion and earlier development of infections as compared with the corresponding groups in Fig. 5d. Interpreting these results in terms of receptor regeneration it appears that regeneration to a level sufficient for infection with this inoculum is considerably more rapid in the presence of calgon, presumably due to inhibition of the residual RDE. It was shown in actual tests on samples from these embryos that immediately after washing out with calgon-saline the fluid in the allantoic cavity had no RDE activity as determined by its capacity to destroy the receptors for MEL virus on fowl red cells. Samples taken after incubation for a further 24 hours showed slight detectable RDE activity.

Comparing the three groups (Fig. 5e) in which the interval between washing out of RDE and inoculation of virus was varied, it will be seen that delay in the development of infection was greatest in the group inoculation immediately after removal of RDE. When a 24-hour interval was allowed, detectable infections occurred approximately 12 hours earlier, while with a 48-hour interval there was no further reduction of the delay in development of infection.

In order to determine whether the characteristic behaviour of IAN O virus in these experiments was a peculiarity of the O phase, limited investigations along the same lines were made with the D phase of the same strain (Fig. 5f). As with the O phase, infection with a large inoculum of IAN D was completely prevented with 0·5 ml. of 4Z agar extract while washing out of the RDE with Ringer solution resulted in late infection in a small proportion of the embryos only.

*Toxicity of RDE for Chick Embryos.*

In the foregoing experiments there was no evidence of any toxic effect of RDE on the embryo when injected into the allantoic cavity in amounts up to 3,200 units. The study of the toxicity by the intravenous route has been complicated by toxic effects apparently due to an associated substance in most of the RDE preparations.

In an attempt to obtain a preparation of RDE free from this associated toxic agent a series of four successive adsorptions and elutions of RDE on red cells was carried out. When the "fourth" eluate (titre 1,000) thus prepared was inoculated intravenously (200 units per embryo) all embryos of a batch of eight were alive 5 days after inoculation and examination at this time revealed no abnormalities.

## DISCUSSION.

These findings concerning the modification of the susceptibility of the tissue cell to virus infection can best be interpreted on the basis of the hypothesis first put forward by Hirst (1943). In discussing his experiments on the adsorption and elution of virus in the excised ferret lung, Hirst postulated that "destruction of a specific receptor substance—which may involve an enzymatic reaction—may be a necessary preliminary event in the parasitism of susceptible cells by influenza virus." In the light of the present work it would seem that this "specific receptor substance" of the susceptible tissue cell, like that of the red cell, can be destroyed or modified by the receptor-destroying enzyme of *V. cholerae* so that the cells are no longer capable of adsorbing virus or susceptible to infection with virus.

In the technique used for demonstration of the action of RDE in preventing allantoic infection, the enzyme was present in the allantoic fluid throughout the course of the experiment, and therefore in continual contact with the allantoic cells. If, on the other hand, the enzyme was removed by washing out the allantoic cavity, the embryo fairly rapidly regained its susceptibility to infection. On the above hypothesis this result is interpreted as being due to regeneration of the receptor substance on the living cells of the allantois. The term regeneration is used in a general sense only, without any attempt to define its mechanism.

In embryos containing RDE it is suggested that regeneration of the receptor substance is occurring continually and that the "concentration" of receptors existing at any one time and therefore the susceptibility of the embryo to infection depends on the balance of the regeneration rate and the rate of enzymic destruction. After an initial phase in which rapid destruction probably takes place unopposed owing to a lag in the commencement of regeneration, an equilibrium would be established between the two opposing processes. The rate of RDE action and hence the level, in terms of receptor concentration, at which this equilibrium is established would depend on the concentration of RDE.

Thus with a small amount of RDE the receptor concentration would not be reduced to a low level even though a long period of time were allowed for the action of the enzyme on the cells before the inoculation of virus. This contention is in accordance with experimental findings. For instance, the results in Fig. 3c show that 160 units of RDE were no more effective in preventing infection with IAN O when given 24 hours before virus instead of 30 minutes before.

Although experiments carried out with different strains of virus cannot justifiably be subjected to accurate quantitative analysis, it is obvious that considerable variation exists in the ease with which RDE prevents infection with the different strains. IAN and CAM, strains of influenza A virus recently isolated, appeared to contrast sharply with the other influenza strains tested, in that the susceptibility of the embryo to infection could be destroyed with much smaller amounts of RDE than were needed for inocula of comparable size of the other strains. This may be interpreted as indicating that IAN and CAM require for growth in the allantoic cavity a much higher concentration of the virus receptors than the other strains. Similarly, the receptor concentration adequate for infection with any one strain of virus varies directly with the size of the virus inoculum. By way of illustration of this concept, four inocula may be arranged in order thus: IAN 10 $ID_{50}$, IAN $10^3$ $ID_{50}$, MEL 10 $ID_{50}$, MEL $10^3$ $ID_{50}$, corresponding with the increasing amount of RDE required to prevent infection and hence with the increasing broadness of the range of receptor concentration over which infection can occur.

Certain conclusions regarding the time required for regeneration of receptors can be drawn from the graphs in Fig. 5. After the washing out of RDE with Ringer solution, regeneration to a level sufficient to allow growth of MEL or SW virus (ca. 10 $ID_{50}$) occurred very rapidly. There is an indication (Fig. 5a) of a slight lag only in the onset of infection, but the results would be compatible with a delay of anything up to 6 hours. The delay in the onset of infection of similarly treated embryos with IAN O ($10^3$ $ID_{50}$), on the other hand, was considerably longer (Fig. 5d). A rough estimate of 54 hours has already been given. It would seem here that the regeneration of receptors to the level necessary for infection with IAN was retarded by the activity of residual RDE as, in the presence of the RDE-inhibitor, calgon, the regeneration time was considerably reduced (Fig. 5e). The fact that embryos in which a 48-hour interval was allowed for regeneration (in the presence of calgon) before inoculation of virus still showed a delay in the onset of infection approximately equal to that shown by the group allowed a 24-hour interval (12–18 hours) probably means that total regeneration does not occur at least in a matter of a few days.

When RDE remained in the allantoic cavity for 24 hours instead of the standard 30 minutes before being washed out (Figs. 5a, 5b and 5c), there was a greater delay in the onset of infection with MEL or SW. This was the only case in which it appeared that RDE might produce more extensive receptor damage when given longer than 30 minutes in which to act.

Investigations reported in this paper are being extended to include a study of the prevention of influenza virus infection in mice. Preliminary results indicate that, as in chick embryos, infection can be prevented with adequate amounts of RDE and that the amount required varies with the strain of virus. These findings, together with the recent evidence that mucin forms the substrate for this enzyme of *V. cholerae* (Burnet, McCrea and Anderson, 1947), may be considered as providing a new basis for a rational approach to the problem of antiviral therapy, either by the use of enzymes with this type of action or of substances which would block the action of these enzymes. It is interesting in this regard to note the recent report by Green and Woolley (1947) that the complex carbohydrate, apple pectin, inhibits virus multiplication in the chick embryo.

## SUMMARY.

The enzyme of *V. cholerae* which destroys the capacity of red cells to agglutinate with viruses of the mumps-influenza group also destroys the ability of the allantoic cells of the chick embryo to adsorb influenza virus haemagglutinin.

Allantoic injection of the receptor-destroying enzyme prevents the development of infection in all or a proportion of embryos following inoculation of virus by the same route. There was considerable variation in the extent of protection afforded by the enzyme against infection with different strains of virus.

The enzyme has no lethal action on the virus particle. Its action in preventing infection is interpreted as due to destruction of a specific receptor substance, on the surface of the tissue cell, with which virus reacts in the initiation of infection. It is suggested that a variation exists in the requirements, in terms of receptor concentration, for infection with different strains.

Evidence is presented that at least partial regeneration of cell receptors can take place subsequent to the action of the enzyme.

The enzyme was non-toxic for chick embryos when injected allantoically or intravenously.

*Acknowledgment.* I am greatly indebted to Professor F. M. Burnet for much helpful advice in the course of this work.

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# THE CONSTANCY OF CHEMICAL COMPOSITION AND INFECTIVITY PER UNIT WEIGHT OF TOBACCO MOSAIC VIRUS PROTEIN PREPARED OVER A PERIOD OF YEARS[1]

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One of the best criteria of the purity of a product is the constancy of composition of samples prepared at different times. However, for a protein the percentage of carbon, hydrogen and even nitrogen may remain reasonably constant, even though considerable foreign protein is present. Phosphorus is, therefore, a better test element on which to judge the purity of a protein containing it.

Much of the literature on the chemical behaviour of plant viruses is reduced in value by the omission of chemical data on the preparations used. Where possible this information has been supplied by the author at the time of publication, but the present data will serve the additional purpose of filling in any gaps, as the work described in previous papers has been done on samples of virus described in this paper or on samples of comparable purity and with similar properties.

The present paper records the phosphorus content of samples prepared over a period of ten years and the content of nitrogen, sulphur, ash, hydrogen and carbon of samples prepared over a period of five years.

When dealing with substances like mosaic virus, which although organized at the molecular or chemical level also behave like a living organism, an additional check on homogeneity and purity is possible and necessary—namely, the infectivity per unit weight. This has been determined for seventeen samples of virus harvested at intervals over a period of nine years.

## EXPERIMENTAL METHODS.

*Preparation of Virus Samples.*

Virus was prepared from juice expressed from artificially infected plants of *Nicotiana tobaccum*, var. Blue Pryor, raised in an insect-proof glass-house by methods previously described (Best, 1940). The virus itself is a direct descendant of a sample presented by Professor James Johnson of the University of Wisconsin to Mr. Geoffrey Samuel in 1930, and was at that time known as Johnson's tobacco mosaic virus No. 1, or "ordinary" tobacco mosaic virus. For all samples prepared after 1938, the "standard" method was used. This consisted in repeated re-precipitation with 15 p.c. ammonium sulphate solution, followed by

[1] Glasshouse facilities and part of the cost of these investigations were provided by the Council for Scientific and Industrial Research.

repeated re-precipitation from an aqueous solution at the iso-electric point of pH 3·4 with 0·04 M acetic acid-acetate buffer. Where dry preparations were required, the final treatments with absolute alcohol and acetone were used. All samples were clean white powders. Samples prepared in 1938 were precipitated from juice held for two hours at about 50° C. instead of being aerated at room temperature only as in the "standard" procedure used for all subsequent preparations.

*Sample 1936/IE.* In the preparation of sample 1936/IE iso-electric precipitation alone was used (Best, 1936). This sample was precipitated in 1936 from juice clarified by centrifuging at about 2,000 *g.* by adjusting the pH value to 3·4, collecting the precipitated virus by centrifuging, and eluting the virus with dilute acetic acid at a pH value just below 3. This gave a clean white preparation which has been stored in suspension at 2° C. ever since. For the purpose of the analyses and the infectivity tests reported in this paper, which were carried out some years later, virus was re-precipitated iso-electrically twice from aliquots of this suspension before being used.

All preparations were stored at 2° C. in pyrex bottles. All except 1936/IE were stored as aqueous solutions at pH 7 and at a concentration of virus between 0·5 and 1 p.c. on a dry weight basis.

*The Analyses.*

Nitrogen was determined by micro-Kjeldahl or micro-Dumas methods or both, phosphorus by the Fisk-Subarrow colourimetric method or the Pregl micro-method, or both. The determinations of carbon, hydrogen, sulphur and ash and some of the estimations of P and N reported in this paper were made by Dr. Georg Burger at the Chemistry Department of the University of Adelaide, using the usual Pregl micro-analytical techniques.

## EXPERIMENTAL RESULTS.

### TABLE 1.

*Showing the elemental composition of samples of tobacco mosaic virus prepared over a period of five years.*

| Sample. | Extracted from. | Percentage composition. P. | N. | S. | Ash. | H. | C. |
|---|---|---|---|---|---|---|---|
| 1936/IE | all leaves | 0·52 | 16·67 | 0·17 | 1·97 | 7·00 | 51·04 |
| 1938/A | all leaves | 0·52 | 16·89 | 0·20 | 1·45 | 7·10 | 51·29 |
| 1938/B/R | roots | 0·54 | 16·76 | 0·17 | 0·78 | 7·08 | 51·94 |
| 1938/B/T | top leaves | 0·51 | 16·44 | 0·15 | 1·22 | 6·92 | 51·74 |
| 1938/C/T | " " | 0·53 | 16·64 | 0·14 | 1·15 | 6·98 | 51·92 |
| 1938/F/T | " " | 0·55 | 16·86 | 0·18 | 1·31 | 7·00 | 51·00 |
| 1939/III/T | " " | 0·50 | 16·53 | 0·12 | 1·32 | 6·85 | 51·75 |
| 1939/III/M | middle leaves | 0·51 | 16·70 | 0·25 | 1·20 | 7·06 | 51·6 |
| 1940/5 | all leaves | 0·53 | 16·25 | 0·17 | 0·94 | 7·01 | 51·41 |
| 1940/10 | " " | 0·50 | 16·51 | 0·15 | 1·12 | 6·89 | 51·92 |
| *Mean of all:* | | 0·52 | 16·62 | 0·17 | 1·25 | 6·99 | 51·56 |

*Note*: The description of the samples in column 1 gives first the year of harvesting and then the number of the harvest for that year (expressed as an ordinary or Roman numeral or a letter) and in some cases a letter to indicate the part of the plant from which the virus was extracted (e.g. T for top leaves, R for roots, and so on).

*Infectivity.*

Solutions of from 0·5 to 1 p.c. of pure virus standardized on a dry weight basis were used at various times to make up solutions in composite buffer of pH 7 containing $1 \times 10^{-6}$ gm. virus (dry weight) per ml. of inoculum. The relative infectivities of these inocula were tested against each other by inoculation on to leaves of *Nicotiana glutinosa* plants, using the opposite half-leaf method, combined with incomplete block or Latin square arrangements. (Samuel, Best and Bald, 1935; Best, 1937a). Most of the samples were tested against each other a number of times. A freshly prepared sample was normally included as a control; and the controls were checked against each other by including them as "tests" in other series.

In addition to the analyses reported in Table 1, a number of other samples have been analysed for nitrogen and phosphorus, including samples prepared in 1944. In all cases when the samples are as pure as we can get them, the nitrogen and phosphorus contents agree (within the experimental error of the methods) with the mean values of Table 1, viz. 16·6 p.c. nitrogen and 0·52 p.c. phosphorus.

TABLE 2.

*A comparison of the infectivity per unit weight of preparations of pure tobacco mosaic virus harvested over a period of nine years. (The numbers in column 3 refer to the infectivity of the sample entered in column 1 expressed as a percentage of the sample entered in column 2 when the weight of virus (dry basis) was $1 \times 10^{-6}$ gm. per. ml. in all systems).*

| Virus sample under test. | Control Sample. | Infectivity of test as p.c. of control. | Time of year harvested. | Duration of infection (days). |
|---|---|---|---|---|
| 1936/IE | 1940/10 | 107 | March | — |
| 1938/A* | 1940/12 | 111 | January | 46 |
| 1938/A* | 1940/10 | 84 | January | 46 |
| 1938/B/R | 1940/10 | 78 | March | — |
| 1938/C | 1940/10 | 99 | June | — |
| 1938/F/T* | 1940/10 | 77 | October | 32 |
| 1938/F/T* | 1940/10 | 89 | October | |
| 1939/VI/T | 1940/10 | 99 | December | 33 |
| 1939/VI/B | 1944/1 | 87 | December | |
| 1940/5 | 1940/10 | 96 | March | 42 |
| 1940/10* | 1940/10 | 125 | July | 41 |
| 1940/10* | 1940/10 | 85 | July | |
| 1941/C | 1940/10 | 88 | February | 34 |
| 1941/D | 1944/1 | 126 | March | 39 |
| 1941/E | 1944/1 | 91 | April | 40 |
| 1941/F | 1944/1 | 102 | April | 40 |
| 1942/2* | 1944/1 | 95 | April | 38 |
| 1942/2* | 1944/1 | 107 | April | |
| 1942/2* | 1944/1 | 106 | April | |
| 1942/2* | 1944/1 | 106 | April | |
| 1944/2* | 1944/1 | 95 | December | 43 |
| 1944/2* | 1944/1 | 77 | December | |
| 1944/2* | 1944/1 | 94 | December | |

* Where more than one entry is made for the same sample, this means that comparisons were repeated at different times as a further check.

## DISCUSSION.

An examination of Table 1 reveals a high degree of uniformity in the analytical results, particularly in the more important elements, phosphorus and nitrogen. The values for hydrogen and carbon also vary only over a comparatively narrow range. The values recorded for the above elements by the author in an earlier paper (Best, 1937b) are in close agreement with those published here for a much wider range of preparations. While differing somewhat from the earlier values published by Stanley and co-workers, they are in good agreement with the later values of these workers and with those of Bawden and Pirie. (For references and summary see Bawden, 1943.)

The mean value of 1·25 p.c. for the ash content of the virus is much lower than the 2·0 p.c. selected by Bawden (1943) as the most probable value. From the amount of NaOH required to bring the virus from its iso-electric point of pH 3·4 to pH 7 the author has calculated an "equivalent weight" of 8,000 for the virus. Allowing for the conversion of all the sodium to $NaPO_3$ during ashing, and of the remaining P to $P_2O_5$ the ash content would be 1·5 p.c. of the virus. On the basis of a simple and complete conversion of the 0·52 p.c. P to $P_2O_5$ the ash from this source would amount to 1·19 p.c.

The values recorded for sulphur by other workers vary from nil to 0·7 p.c., and Bawden (1943) selects a value of 0·4 p.c. as the most probable one. The values for sulphur recorded in Table 1 fall within a much narrower range than those recorded by others, but even so they show too great a variation to be regarded as more than approximate. The mean value of 0·17 p.c. recorded in Table 1 is close to the value previously recorded by Best and Lugg (1944) of 0·20 p.c. sulphur for a particularly pure preparation of the virus. In view of the method used, this latter value may be taken as the best yet obtained, particularly as it agrees with that of Ross (1940), who recorded values ranging from 0·17 to 0·20 p.c. for his best preparations and regarded 0·20 p.c. as the most probably correct value.

### *Infectivity.*

Of the seventeen samples (including controls) which were tested against each other (Table 2), none differs significantly from any of the others in respect to infectivity per unit weight of virus. This conclusion holds not only for samples prepared within a few months of each other, but also for samples differing in age by over four years.

Analytical figures and infectivity tests seem to indicate that the author's preparations gathered over a period of years are more uniform and consistently purer than most described in the literature, so that it is not surprising that many of the discrepancies noted by others have not been noticed in these laboratories. Bawden and Pirie (1938) have done a considerable amount of work on proteins

present in the juice expressed from healthy tobacco and other plants. From their data it is clear that the techniques used by the author for separating the virus should be very effective in eliminating non-viral proteins. The analytical and infectivity figures recorded here bear this out. But it must be remembered that ordinary tobacco mosaic virus gives rise by mutation to small quantities of at least one other related, but distinct, strain of similar chemical composition. Although such strains would be present in only small amounts, all preparations of tobacco mosaic virus, including those described in this paper, may be expected to contain some of them.

## SUMMARY.

Ten samples of ordinary tobacco mosaic virus harvested at various times over a period of five years had the following percentage composition:

| | P | N | S | Ash | H | C |
|---|---|---|---|---|---|---|
| Range | ·50–·55 | 16·25–16·89 | ·12–·25 | ·78–1·97 | 6·85–7·10 | 51·00–51·94 |
| Mean | 0·52 | 16·6 | 0·2 | 1·2 | 7·0 | 51·6 |

Infectivity tests of seventeen samples of the virus harvested over a period of nine years showed that there was no significant difference between the infectivity per unit weight.

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# THE MUCINASE OF *V. CHOLERAE*

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Despite the importance of mucinous secretions in physiological and pathological conditions there has been strikingly little recent study of the mucins produced by mucous glands and mucous membranes. This contrasts with the considerable attention that has been paid to the mucins of connective tissue synovial fluids, etc. The interest in the mucins which contain hyaluronic acid and its polymers has arisen largely from the investigations of the spreading factor of Duran-Reynals and its identification at least in part with the enzyme (or group of enzymes) hyaluronidase. The work to be described has been undertaken from the belief that the existence of an active enzyme which destroys the characteristic properties of glandular mucin might similarly open up an active field of research on mucin of this type. As is well known, hyaluronidase has no action on the ordinary types of glandular mucin, and as will be shown in this paper, vibrio mucinase is without action on hyaluronic acid protein mixtures.

In a previous paper (Burnet and Stone, 1947) the initial work which led to the recognition of a mucinase in culture filtrates of *Vibrio cholerae* has been described. There it was shown that *in vitro* desquamation of epithelium from inverted segments of guinea-pig intestine by such filtrates was essentially due to the presence of a mucinase. It was also shown that antiserum against the enzyme could be prepared by appropriate immunization of rabbits.

Concurrent work on what we have called the RDE (receptor-destroying enzyme) of *Vibrio cholerae* has shown that this enzyme also acts on glandular mucin. The two enzymes apparently attack two different aspects of the mucin molecule or possibly two different molecular types present together in crude and semi-purified mucins. The present paper is concerned only with the enzyme or group of enzymes responsible for changing the physical character of mucin solutions. It has been possible to produce from *V. cholerae* filtrates preparations of high titre mucinase with no RDE activity and others with full RDE activity and very low mucinase effect. All the evidence suggests that two wholly distinct enzymes are concerned in the two reactions.

The present communication is of a preliminary character. From the nature

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of the enzyme, work could only be carried out with native mucin and our attempts to purify crude solutions by appropriate precipitation have always resulted in preparations lacking the essential physical qualities of native mucin. Apart from a number of dialyzed preparations made to study the action of salts on the reaction, all the mucin preparations used have been in the form of simple dilutions of physiological or pathological mucin-containing secretions. Most of the experiments, too, were made with vibrio filtrates obviously containing other enzymes. Nevertheless, we believe that using the test described for intact mucin and for its destruction, we have been concerned with a single enzyme-substrate reaction, and that the results obtained merit being reported.

In the course of the work, a simple practical test for the presence of intact mucin, either of glandular or of connective tissue type, has been developed. This can be used for the titration of any type of enzyme with a depolymerizing effect on either mucin. In many respects, this paper can be regarded essentially as an exploration of the potentialities of this test.

## EXPERIMENTAL.

*Preparation of Vibrio Mucinase.*

The method used has been described previously (Burnet and Stone, 1947). It consists in extracting the fluid from 16 to 24 hour cultures of *V. cholerae* on soft agar plates with subsequent centrifugation and filtration through a Seitz disc. Most of the experiments to be reported were made with such crude filtrates which also contained RDE and presumably a variety of other enzymes as well as mucinase. Recently a simple method of eliminating RDE without damage to the mucinase has been developed. This arose from the observation that some cultures and not others, when incubated for 24 hours instead of the usual 16–18 hours, showed very low RDE titres. Investigation showed that these low titres came from plates which had developed a higher alkalinity than those giving potent enzyme. If a fresh active filtrate is adjusted to pH 8·5 and held at 37° C. for 4 hours, all RDE activity disappears without loss of mucinase activity. The conditions of this destruction of RDE have not yet been worked out in detail, but it appears likely that the alkaline reaction allows destruction of RDE by some other enzyme.

Limited experiments on the purification of mucinase have shown that it is precipitated by saturation in the cold with ammonium sulphate and after dialysis can be precipitated by two volumes of acetone in the cold. Such preparations, however, also contain RDE and in general the receptor-destroying enzyme is recovered in better yield than the mucinase. Filtrates in which RDE has been inactivated by incubation at pH 8·5 can be similarly treated to give dried semi-purified material with no RDE activity.

Apart from investigations previously reported (Burnet and Stone, 1947), no

attempts have been made to prepare mucinase from other types of bacteria than *V. cholerae.*

*Sources of Mucin and Preparation of Solutions.*

In order to obtain satisfactory experimental results, it is necessary to have a homogeneous solution or suspension of mucin in saline. Most of the experiments have been carried out with guinea-pig intestinal mucin obtained as follows: Guinea-pigs are anaesthetized and a laparotomy performed to expose the lower end of the ileum. The last 20 cm. of the ileum are tied off and unless empty are washed out with saline. The openings are then sutured and sufficient normal saline injected into the lumen to distend the bowel firmly. The abdomen is closed and the animal kept under sedative for 2 to 3 hours. It is then killed and the mucinous fluid now filling the distended segment withdrawn with a syringe and a wide-bore needle. The fluid is usually clear and homogeneous, moderately viscous and generally free from blood or food residues.

A good deal of work has been done with the contents of a cyst of the human ovary removed at operation. Two other similar cyst contents and peritoneal fluid from a case of pseudomyxoma peritonaei contained only pseudomucin which is unsuitable for this type of work. The cyst in question, which was obtained through the co-operation of Dr. Ella McKnight and Dr. Marion Wanliss, contained in addition to much brownish pseudomucinous material, a locule filled with very tenacious white mucus and also strings of similarly tenacious brownish material in the main body of the cyst. This denser material was separated from the syrupy pseudomucin and proved very satisfactory for a number of types of experiments. It was not easy to homogenize directly at the required dilution, but if a millilitre was removed from the mass and placed in 10 ml. of diluent, either distilled water or saline, sufficient water was imbibed to allow easy homogenization the following day. Rapid up and down movement in a teat pipette with a wide opening was found to be the most practical means of homogenization.

The mucin from the fasting gastric contents of achlorhydric patients varies greatly in its physical qualities, but sufficient numbers of samples with a relatively high concentration of clear viscous mucin were obtained to allow a fairly extensive study. The contents were neutralized if necessary and homogenized in the same way as the ovarian mucus. The keeping qualities of gastric mucin appeared quite unpredictable: some specimens contained a demonstrable mucolytic enzyme and rapidly lost viscosity, others without demonstrable action on other specimens also deteriorated similarly, while a few remained active for weeks in the refrigerator.

Several samples of uterine cervical mucus were also used. Despite the initial dense untractable appearance of the specimens, it was possible to produce satisfactorily homogeneous solutions which were susceptible to attack by vibrio mucinase.

*Technique of Mucinase Titrations.*

After testing various possibilities for titration methods, an attempt was made to devise a method based on the firm coagulation of mucin that was observed when Carnoy's fluid was used as a fixative for intestinal segments. If a mucin solution is dropped into acid alcohol, a firm pellicle forms at the interface, maintaining the drop either in globular or with weaker solutions in annular form. The shape can be rendered easily visible by adding an alcohol-insoluble dye to the mucin solution. In the absence of mucin the drop flattens out immediately and leaves only a cloudy patch without a definite margin. The method is very quick and results reproducible within the required order of accuracy, provided a standard preparation of mucinase is included in each set of tests.

Details of the method of mucinase titration are as follows:

The mucin solution to be used as substrate is first thoroughly homogenized by drawing up and down in a pipette. Serial two-fold dilutions are made in saline buffered with borate at pH 7 in 0·5 ml. quantities. To each tube two drops of 0·1 p.c. Congo red solution in water are added. One drop from each tube is then dropped gently into a shallow layer of acid alcohol (4 mm. ±) in a flat dish with transparent bottom—either a petri dish or a similar shaped vessel turned from transparent plastic. The acid alcohol is a 1 p.c. solution of strong hydrochloric acid in 70 p.c. alcohol. The shapes taken by the drops are recorded by a suitable convention. With falling dilutions one obtains first a compact lenticular "blob" ++, then an annular form with a characteristically corrugated internal boundary +±, the next stage is that which just maintains the annular form with an intact well defined outer boundary +. At further dilutions the pattern becomes progressively imperfect and we have chosen as end-point a ± reaction which shows temporary formation of an annulus which immediately disintegrates peripherally, but leaves an incomplete streak of stained mucin to mark the position of the inner margin of the annulus. Finally, dilutions beyond a certain point give only a rapidly dissipating diffuse haze. A typical result of such a preliminary titration of mucin is shown in Table 1.

From this titration the dilution which will give a + reaction is chosen and a stock solution prepared at three times the concentration. When this is diluted 1:2 in the test the control tubes will give a reaction better than + and allow the end point of mucinase action to be clearly seen. We have found it desirable to use what is actually the smallest amount of mucin that can safely be used rather than a larger amount, both from reasons of economy and because of the sharp changes in apparent titre with different substrate concentrations which are shown by some types of mucin (v. infra).

Serial dilutions of the enzyme to be titrated are prepared in buffered saline and an equal volume of stock mucin solution added (total volume 0·5 ml.). The racks are placed in a 37° C. bath for 1 hour. Readings are made as in the preliminary titration and the end point of mucinase activity taken as the ± reading which has been described. A standard mucinase is always included in the titration. This is a crude agar filtrate of high titre which has been mixed with an equal volume of pure glycerol and stored at —10° C. A 1:1,000 dilution from this stock is used to start the titration series.

In the absence of any convenient short way of referring to this technique we have called it the ACRA test (C-ongo R-ed in A-cid A-lcohol).

TABLE 1.

*Titration of mucin, mucinase and antimucinase to illustrate general technique.*

*Preliminary mucin titration.*

| | Mucin dilutions. | | | | |
|---|---|---|---|---|---|
| | 5. | 10. | 20. | 40. | 80. |
| G P 50 | ++ | ±± | + | ± | — |

*Mucinase titration using 1:6 dilution of mucin.*

| Enzyme preparation. | Enzyme dilutions. | | | | | | | Titre. |
|---|---|---|---|---|---|---|---|---|
| | 100. | 200. | 400. | 800. | 1,600. | 3,200. | 6,400. | |
| Standard | — | — | — | — | tr | + | + | 2,400 |
| Agar Filtrate 7 | — | — | — | — | ± | + | + | 1,600 |

*Antimucinase titration using 1:20 AgF7* i.e. *80 doses of mucinase.*

| Serum. | Serum dilutions. | | | | | | | Titre. |
|---|---|---|---|---|---|---|---|---|
| | 10. | 20. | 40. | 80. | 160. | 320. | 640. | |
| R 148—N | — | — | — | — | — | — | — | <10 |
| R 148 3 | + | + | + | + | ± | — | — | 160 |

++, +, ± diminishing degrees of cohesiveness of pattern in acid alcohol as described in the text.

This Table illustrates the use of a guinea-pig intestinal mucin as substrate in mucinase and antimucinase titrations. R 148—N and R 148 3: initial and post-immunization serum from a rabbit intensively immunized with *V. cholerae* filtrates.

### *Application of the ACRA Technique to Titration of Hyaluronidase.*

Hyaluronic acid as prepared by the method of McClean (1943) does not produce a defined pattern by the method, but a suitable substrate can readily be prepared from human synovial fluid. Ten ml. of saline are injected into the knee joint of a cadaver and as much fluid as possible subsequently withdrawn. This fluid can be diluted 20 or 40 fold before the limiting "+" reaction by the ACRA test is reached.

Hyaluronidase can be titrated in exactly the same fashion as titrations of cholera mucinase or glandular mucin. The technique is far simpler than any viscosimetric procedure and in our opinion easier to read than the mucin clot method. For ordinary bacteriological work where convenience is more important than a high degree of accuracy, it is probably the method of choice.

*The Characteristics of Vibrio Mucinase as Tested on Guinea-Pig Intestinal Mucin.*

The general features of the reaction can be stated briefly as follows:

(1) The activity is not significantly affected between pH 7·0 and 8·6. Below 6·5 the pattern is ill-defined and the end-point unreadable, and above 9·0 the apparent titre of a mucinase diminishes sharply. Normally, dilutions are made in borate buffered saline at pH 7.

(2) Salts must be present for the reaction to occur, but it appears to be relatively unimportant what ions are present. Dialyzed preparations of mucin and mucinase show practically no activity. With M/250 NaCl as diluent, a titre of 1,000 was obtained with a mucinase preparation that gave a titre of 1,600 with standard M/15 buffered saline. Phosphate or calcium chloride solutions of similar molarity were also effective.

(3) The mucinase activity is slightly higher in the presence of Ca ions than in their absence, but there is no regularity in the inhibitory effect of Ca deionizing agents. Citrate and oxalate produce a distinct inhibition, but fluoride at M/20 has no effect. By far the most potent inhibitor we have found is sodium hexametaphosphate "Calgon" which is completely inhibitory at M/60.

(4) With guinea-pig mucin the titre obtained for a given mucinase preparation is inversely proportional to the concentration of mucin used as substrate. Deviations from this finding are sometimes encountered with guinea-pig material and the extreme deviation found with mucin of other types is referred to later. Table 2 includes a protocol of a typical experiment.

*The Influence of Substrate Concentration on Mucinase Activity.*

In testing a wide variety of mucinous fluids, considerable differences have been observed in their rheological character, the type of pattern produced in the ACRA test and the influence of concentration used on the apparent titre of a standard mucinase preparation.

The primary difference appears to be between mucin and pseudomucin. Pseudomucinous fluids are viscous with a certain degree of "spinnbarkeit" but are usually almost homogeneous. If sufficiently concentrated they produce a solid "blob" in acid alcohol, but with very slight dilution the pattern loses its edge without showing the sequence of changes characteristic of true mucin on dilution. It is not practicable to carry out mucinase titrations and it seems probable that pseudomucin is actually the result of enzymic or other processes acting on mucin in similar fashion to the vibrio mucinase.

Guinea-pig mucin obtained as described is generally similar in obvious physical character to pseudomucin but has a greater degree of "spinnbarkeit." It produces well-defined patterns in the ACRA test and is highly sensitive to vibrio mucinase. When different concentrations of the same fluid are used in parallel the mucinase titres obtained are to the degree of accuracy of the test inversely proportional to the concentration used.

Fasting gastric contents vary widely in the character of the mucin present. A few have contained pseudomucin. Most contain highly viscous-elastic material which is homogenized with difficulty but eventually gives uniform solutions. Typical patterns in acid alcohol are produced by successive dilution. The most striking characteristic of such preparations is the sharp influence of concentration on the mucinase effect. If final dilutions giving the limiting "+" pattern are used, the titre of a standard mucinase is very close to that obtained with a similarly diluted guinea-pig mucin. But if stronger concentrations are used, the mucinase becomes disproportionately less active. If an hour's contact between mucinase dilutions and substrate is allowed before reading, one may obtain a titre of say 2,000 with "+" concentration and only 1 : 10 with a two-fold higher concentration. The discrepancy diminishes somewhat on further contact, but remains in quite striking contrast to the results with guinea-pig intestinal mucin.

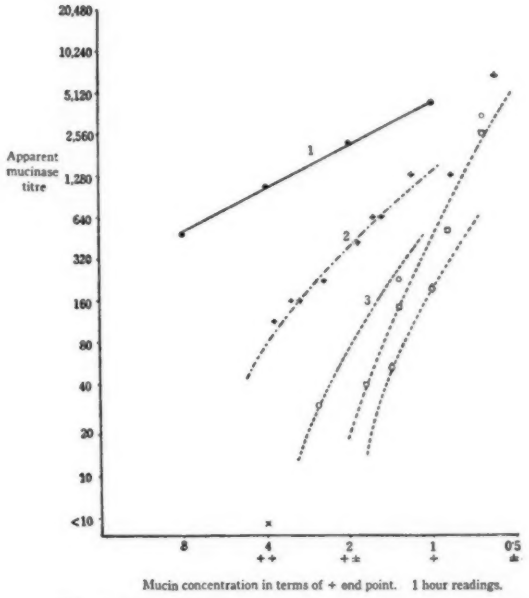


**Fig. 1.** The influence of substrate concentration on the end point obtained in titration with standard mucinase.

1. Guinea-pig intestinal mucin. 2. Ovarian cyst mucin (three experiments). 3. Three specimens of human gastric mucin.

The mucin concentration is shown in terms of the concentration giving + level by the ACRA test.

Comparative results of such experiments are given in Table 2, and in Fig. 1 are collected the results from a number of different types of mucin to illustrate the differences of behaviour.

Mucinous material from an ovarian cyst gave all the typical reactions of intact mucin, was readily homogenized and showed changes in mucinase titre with substrate concentration of intermediate type.

*Characteristics of Hyaluronidase Action as Determined by ACRA Test.*

A number of experiments were carried out on the activity of hyaluronidases prepared from cultures of *Cl. perfringens* and *Staphylococcus aureus* primarily to

compare the process with that of vibrio mucinase on glandular mucin. Suitably diluted human synovial fluid was used as the substrate. Table 3 shows the result of a comparative test with staphylococcal preparations whose titre by McLean's mucin clot test was 600. It will be seen that the hyaluronidase titre is nearly inversely proportional to the concentration of synovial fluid used and that the action is limited to this type of mucin. Vibrio mucinase has no action whatever on synovial fluid.

TABLE 2.

*Influence of substrate concentration on mucinase titration.*

*1. Guinea-pig intestinal mucin.*

| | 1 hour. | 2 hours. | 4 hours. | + level. |
|---|---|---|---|---|
| Gp. 48 undiluted | 500 | 1,200 | 2,500 | |
| 1:2 | 1,200 | 2,400 | 5,000 | 1:8 |
| 1:4 | 2,400 | 4,800 | 10,000 | |
| 1:8 | 4,800 | 10,000 | 20,000 | |

*2. Gastric contents mucin.*

| | 1 hour. | 2 hours. | 4 hours. | + level. |
|---|---|---|---|---|
| GR1 1:5 | <20 | <20 | 20 | |
| 1:10 | <20 | 50 | 120 | 1:20 |
| 1:14 | 60 | 160 | 320 | |
| 1:20 | 200 | 640 | 1,000 | |

*3. Ovarian cyst mucin.*

| | 1 hour. | 2 hours. | 4 hours. | + level. |
|---|---|---|---|---|
| QV1 1:20 | 160 | 320 | 500 | |
| 1:30 | 400 | 640 | 1,000 | 1:60 |
| 1:50 | 1,280 | 2,000 | 2,560 | |
| 1:100 | 6,000 | >6,000 | >6,000 | |

TABLE 3.

*Action of staphylococcal hyaluronidase on joint fluid mucin.*

| Substrate. | Hyaluronidase. | Vibrio mucinase. |
|---|---|---|
| Syn F 2 1:20 | 1,200 | <2 |
| 1:40 | 4,000 | <2 |
| 1:60 | 6,400 | <2 |
| Cyst mucin 1:100 | 15 | 400 |

Tests were carried out with dilutions made in saline containing various potential inhibitors with the results shown in Table 4.

TABLE 4.

*Action of inhibitors on staphylococcal hyaluronidase.*

| Diluting fluid. | Hyaluronidase titre. |
|---|---|
| Buffered saline 7·0 | 2,000 |
| Saline + M/60 Calgon | 1,000 |
| Saline + M/20 K.oxalate | 1,000 |
| Saline + M/20 K.fluoride | >100 |
| 0·5% $CaCl_2$ | 2,400 |
| Distilled water | <100 |

The results confirm the findings in the literature on the necessity for salts to be present and the inhibitory effect of fluoride. It is noteworthy that fluoride has no action on vibrio mucinase and that "Calgon" which is an active inhibitor of mucinase is without action on the hyaluronidase.

Dried toxins of *Cl. perfringens*, supplied by Mr. J. F. McCrea, were also active when tested by the present method, the titres obtained with three preparations being 12,000 (240), 8,000 (120) and 16,000 (480), the "mucin clot" titres being shown in parentheses.

A point of interest which we have not seen mentioned in the literature is that the pattern-producing power of synovial fluid is rapidly destroyed by very low concentrations of potassium periodate. If M/10,000 $KIO_4$ is added to synovial fluid at the standard concentration, the power to produce a pattern in acid alcohol is lost within 30 minutes at 37° C.

*Action of Pure Enzymes on Mucin as Shown by ACRA Method.*

Samples of pure trypsin and chymotrypsin obtained through the B.C.S.L.O., Washington, from Dr. J. H. Northropp were tested for their action on the available types of mucin by ACRA method.

TABLE 5.

*Action of trypsin and chymotrypsin on mucin.*

| | Trypsin. | | Chymotrypsin. |
|---|---|---|---|
| | 1 hour. | 4 hours. | 1 hour. |
| Synovial fluid | <100 | <100 | <10 |
| Cyst mucin | 600 | 1,600 | 640 |
| Guinea-pig mucin | <100 | 100 | <10 |

Figures show the dilution of a 1:500 solution of pure enzyme that gave ± destruction of pattern with mucins at standard dilution.

The action on cyst mucin is quite similar to that of the vibrio mucinase. Some samples at least of gastric mucin are sensitive to trypsin and one of six samples of guinea-pig intestinal mucin was acted on. It seems probable that the absence of action is due to the presence of antitryptic factors in the mucin preparation rather than intrinsic insusceptibility of the mucin molecule.

It is possible that for some purposes titrations of trypsin and of antitryptic activity could be very conveniently made by this method.

## DISCUSSION.

The two objects of this paper are, first to draw attention to the existence of an enzyme or group of enzymes produced by cultures of *V. cholerae* which have an activity on glandular mucins analogous to the action of bacterial hyaluronidases on connective tissue mucin and to describe the ACRA test as a convenient method for titrating the activity of both groups of enzymes. In other papers, a second enzyme produced by the cholera vibrio, the so-called receptor-destroying enzyme, will also be shown to act on glandular mucin, but in a wholly different fashion. These two enzymes should become valuable tools for the study of the chemistry and the physiological and pathological activities of glandular mucin.

## SUMMARY.

A simple test is described for the recognition of natural mucins, either of glandular or connective tissue origin: it depends on the pattern produced when dilute solutions of mucin coloured with Congo red are dropped into acid alcohol (ACRA test).

This test has been used in a study of the activity of a mucinase (depolymerase) from *V. cholerae* filtrates. The enzyme is active against a variety of glandular mucins but is without action on human synovial fluids (hyaluronic acid type mucin).

The influence of substrate concentration on the apparent titre of vibrio mucinase varies sharply with the type of mucin used.

The activity of the mucinase is completely inhibited by sodium hexametaphosphate, and like hyaluronidases, it is inert in the absence of salts.

Hyaluronidases can be rapidly titrated by determining the dilution to which the positive ACRA reaction with synovial fluid is destroyed under standard conditions.

Solutions prepared from ovarian cyst mucin can be used for the titration by the ACRA technique of trypsin or chymotrypsin as well as vibrio mucinase.

*Acknowledgment.* I am indebted to Mr. H. F. Holden for the preparation of dried enzyme and for help on other chemical aspects, and to Mr. J. F. McCrea for providing hyaluronic acid and hyaluronidase preparations.

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# THE ACTION OF RENIN AND HYPERTENSIN ON THE PULMONARY AND HEPATIC CIRCULATIONS

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Among the many observations of the action of renin and hypertensin in the various fields of the vascular system, there are no records of their effects on the pulmonary or hepatic circulations. Lewis and Goldblatt (1942) state on the basis of a personal communication from Katz and Rodbard that the pressure in the pulmonary artery is not raised by renin; but the animal used is not indicated.

This paper presents observations of these actions of the renin mechanism.

## METHODS.

Cats, rabbits and dogs were used. All of the animals were anaesthetized with chloralose after preliminary ether anaesthesia. During the course of an experiment the rabbits required a little additional ether, and the dogs had previously been given 15 mg. per kg. of morphine tartrate subcutaneously. Both vagi were divided, the trachea cannulated, and artificial respiration set up. The pressure in the pulmonary artery was recorded by the method used by Kellaway and LeMessurier (1936). In some experiments the pressure in a corresponding pulmonary vein was recorded simultaneously, the cannula in the vein being connected through a saline manometer to a Brodie tambour. The pressure in the portal vein was recorded through a cannula in the splenic vein of the cat and dog, or in a mesenteric vein of the rabbit, the recording set-up being the same as for the pressure in the pulmonary vein. The spleen was removed and the splenic artery isolated for intra-arterial injections. The venous and arterial cannulae were filled with a saturated solution of sodium sulphate and, as a further precaution against clotting, heparin was injected into them. In two experiments the hepatic outflow of blood was estimated by a modification of the method used by Feldberg and Schilf (1930) for measuring the outflow from a hind limb. The aorta was tied below the origin of the superior mesenteric artery; both renal arteries were also tied and the inferior vena cava was occluded with a ligature above the right suprarenal vein. The chest was opened on the right side sufficiently to enable a large transfusion needle to be inserted into the inferior vena cava just above the diaphragm. After its insertion the inferior vena cava was ligated just below the right auricle and the outflow from the needle, consisting almost entirely of hepatic blood, was returned to the heart through a cannula connected to the right external jugular vein. The rate of flow was recorded with a Gaddum's outflow recorder. Coagulation was prevented by means of heparin.

Injections were made into the systemic system through a cannula in a saphenous or external jugular vein, into the portal system through a small tributary of the portal vein and into the hepatic artery through a metal cannula tied into the cut stump of the splenic artery.

Samples of renin and hypertensin were prepared by one of us (H.J.). Renin was prepared

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from the kidneys of dogs, cats, rabbits and pigs by the method of Braun-Menendez, Fasciolo, Leloir and Munoz (1940), or by the method of Helmer and Page (1939). For brevity, these preparations have been designated "renin (B)" and "renin (H & P)" respectively. The latter preparation was taken to the fraction C stage and compared with the former had very much less depressor activity. We prepared hypertensin by the method of Braun-Menendez *et al.* (1940), designated "hypertensin (B)," and in addition, we used a sample kindly supplied to us by Dr. I. H. Page designated "hypertensin (H & P)."

## RESULTS.

### THE PRESSURE IN THE PULMONARY ARTERY.

The effect of various preparations of renin or hypertensin on the pressure in the pulmonary artery of cats, dogs and rabbits is set out in Table 1. The figures in this Table represent the pressure increments in the carotid and pulmonary arteries at the peak systemic pressure. In no instance could the rise of pulmonary pressure be attributable to "back-pressure" from the increased tension in the aorta. This was shown by comparing the rise in pulmonary arterial pressure with the insignificant changes in pressure which occurred in that artery when the aorta was occluded with a spring clip just above the diaphragm, and, in two experiments, by recording simultaneously the pressure in both pulmonary artery and vein.

TABLE 1.

| Animal. | Injection. | Pressure increments at the peak systemic pressure. | |
|---|---|---|---|
| | | In carotid. | In pulmonary. |
| Cat 1 | Renin (pig) (H & P) | 22 mm. Hg. | 3 mm. Hg. |
| 2 | Renin (pig) (B) | 51 " " | 13 " " |
| | Hypertensin (B) | 100 " " | 20 " " |
| 3 | Renin (cat) (B) | 106 " " | 15 " " |
| 4 | Renin (pig) (H & P) | 28 " " | 3 " " |
| | Hypertensin (H & P) | 64 " " | 5 " " |
| | Hypertensin (B) | 64 " " | 5 " " |
| 5 | Hypertensin (H & P) | 31 " " | 5 " " |
| Rabbit 1 | Renin (pig) (H & P) | 24 " " | 4 " " |
| 2 | Renin (pig) (B) | 11 " " | 7 " " |
| | Hypertensin (B) | 13 " " | 8 " " |
| 3 | Renin (pig) (H & P) | 24 " " | 3 " " |
| | Hypertensin (H & P) | 14 " " | 3 " " |
| 4 | Renin (rabbit) (B) | 6 " " | 2 " " |
| Dog 1 | Renin (dog) (B) | 40 " " | 0 " " |
| 2 | Renin (dog) (B) | 31 " " | 0 " " |
| 3 | Hypertensin (H & P) | 25 " " | 0 " " |

*The Cat.*

It is clear from the Table that there was a significant rise of pressure in the pulmonary artery in all of the cats when renin from pig or cat or when hypertensin was injected. Fig. 1 illustrates a typical experiment and shows the effect of an injection of cat renin (B) at R, occlusion of the aorta as a control observation at C and, in a second animal, the effect of an injection of hypertensin (B) at A.

*The Rabbit.*

As in the cat there was a significant rise of pressure in the pulmonary artery.

*The Dog.*

Apart from a small initial rise associated with a preliminary depressor effect, we found, in the dog, no significant change in pressure in the pulmonary artery when renin (B) or hypertensin (H & P) was injected.

THE PRESSURE IN THE PORTAL VEIN.

The intravenous injection of renin caused, regularly, in all three species, a rise of portal pressure. Commonly, hypertensin had a similar action, but for a given rise of systemic arterial blood pressure it caused a smaller rise of portal pressure than did renin. The rise of portal pressure began at the same time as the rise of systemic arterial pressure, but usually continued to rise after the latter had reached its peak. Sometimes a distinct biphasic effect could be observed—an initial small rise beginning with the rise of arterial pressure, and a second, main rise, some 15 to 20 seconds later. Occasionally, with hypertensin, the main rise was preceded, not by an initial rise but by a transient fall in portal pressure beginning at the same time as the systemic hypertension.

Pressure records from the external jugular vein or inferior vena cava showed clearly that the rise of portal pressure was not simply a reflection of a raised systemic venous pressure.

In the cat and rabbit the nature of the rise of portal pressure was explored more fully. In each of these species the results were closely similar, but the change in portal pressure was of greater magnitude relative to the arterial pressure change in the rabbit than in the cat. Also, for a given rise of systemic arterial pressure, adrenaline caused in the cat a bigger rise of portal pressure than did hypertensin, whereas in the rabbit, hypertensin was more effective than adrenaline. In both species, as also in the dog, pituitrin regularly caused a sharp fall in portal pressure, as described by McMichael (1932).

*The Dog.*

Four animals were used. Dog or pig renin (B) caused an initial depressor effect, usually associated with a fall of pressure in the portal vein, followed by a rise in both portal and systemic arterial pressures. Hypertensin had in two of the animals a trivial effect only, which was preceded by a transient and slight fall commencing at the time that the blood pressure began to rise (there being no depressor effect). Observations made previously (Reid, 1946) indicated that in the dog the initial depressor effect of the renin samples is due to a vasodilator substance causing dilatation in the hind limb demonstrable by the method of Feldberg and Schilf (1930).

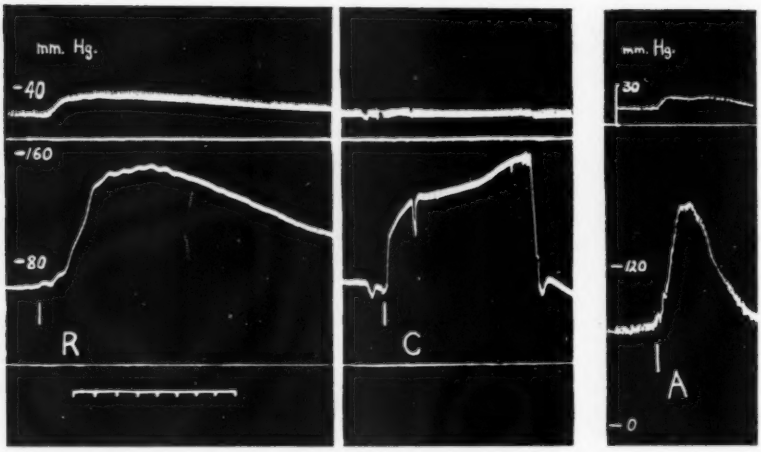


**Fig. 1.** First two panels show the carotid and pulmonary arterial pressures in a cat weighing 2·8 kg. At R, 1·0 ml. of a solution of cat renin (B) was injected and at C the aorta was clamped for a few minutes above the diaphragm.

The third panel shows the pressures in the carotid and pulmonary arteries in a second cat. At A, 1·0 ml. of a solution of hypertensin (B) was injected. Time in half minutes, details in text.

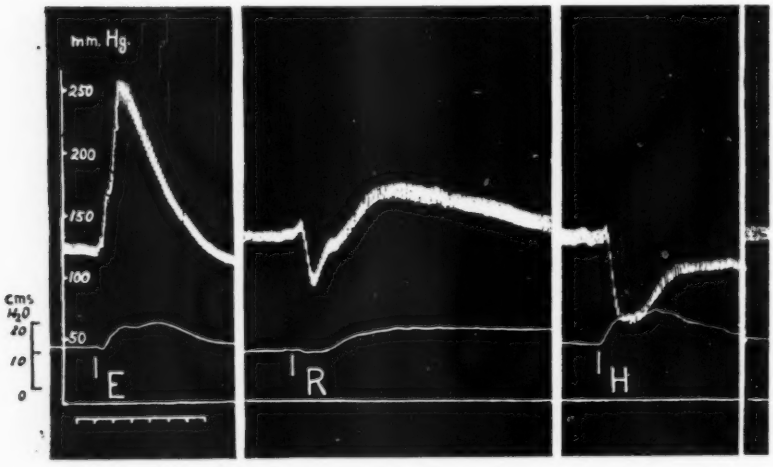


**Fig. 2.** Pressure in the carotid artery and portal vein of a dog weighing 8·5 kg. At E, 30$\mu$g. of adrenaline hydrochloride were injected, at R, 1·0 ml. of a solution of pig renin (B) was injected and at H, 50$\mu$g. of histamine phosphate. Time in half minutes, details in text.

Fig. 2 shows the pressure in the portal vein and carotid artery of a dog. At E 30$\mu$g. of adrenaline hydrochloride, at R 1·0 ml. of a solution of pig renin (B), and at H, 50$\mu$g. of histamine acid phosphate were injected intravenously. It is interesting to note, that the initial depressor effect of this sample of renin is associated with a fall in portal pressure, indicating that the depressor agent is not histamine nor an agent which acts by its liberation.

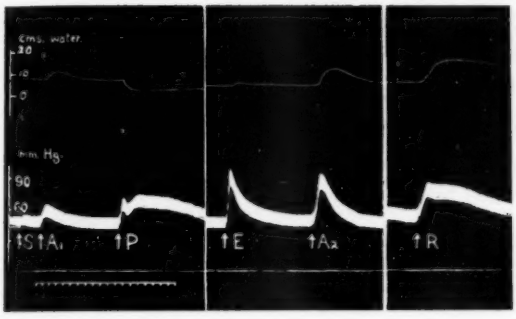


Fig. 3. Pressures in the portal vein and carotid artery of a rabbit weighing 1·5 kg. The following injections were given intravenously: 4·0 ml. of normal saline at S, 0·05 mg. of hypertensin (H & P) at $A_1$, 0·2 units of pituitrin at P, 5·0$\mu$g. of adrenaline at E, 0·1 mg. of hypertensin at $A_2$, and 1·0 ml. of pig renin at R. Time in half minutes, details in text.

*The Rabbit.*

Renin (H & P) and (B), and hypertensin (H & P) and (B), regularly caused a rise of pressure in the portal vein. With an arterial pressure increment of 26 mm. Hg. due to renin (B) the portal pressure rose by 10 cm. of water. In another animal renin (H & P) caused rises of 42 mm. Hg. and 4 cm. of water in the carotid artery and portal vein respectively. With rises of systemic pressure due to hypertensin ranging from 16–50 mm. Hg., the portal pressure rose by amounts ranging from 1·6–9 cm. of water.

The effects of renin and hypertensin are illustrated in Fig. 3. This Figure shows the pressure in the carotid artery and portal vein of a rabbit weighing 1·5 kg. The effects of 0·05 mg. of hypertensin (H & P) at $A_1$, 0·2 units of pituitrin at P, 5$\mu$g. of adrenaline at E, 0·1 mg. of hypertensin (H & P) at $A_2$, and 1·0 ml. of renin (B) at R, are shown. All injections were given intravenously.

*The Cat.*

Renin injections which caused blood pressure increments ranging from 36–94 mm. Hg. produced portal pressure rises of from 2–6 cm. of water. In five animals, hypertensin caused the portal pressure to rise by 1·2 to 3·5 cm. of water, while the arterial rose by amounts ranging from 21–49 mm. Hg. In one of these animals a rise of 2·2 cm. was preceded by a fall of 0·8 cm., coincident with the beginning of the rise of arterial pressure. In a sixth animal the effect on the portal pressure was trivial. In contrast to the rabbit, the effect on the portal pressure was, for a given rise of systemic arterial pressure, less than that due to an injection of adrenaline.

## ANALYSIS OF THE RISE OF PORTAL PRESSURE IN THE CAT AND RABBIT.

### *Effect of Intraportal Injections.*

Intraportal injections of hypertensin caused the rise of portal pressure to begin several seconds before the rise of arterial pressure; but the peak rise of

portal pressure was regularly less than when injections were made into a systemic vein of the same animal. As already described, sometimes a two-phase effect could be seen following intravenous injection. When this took place it was found on subsequent intraportal injection that the small initial rise was exaggerated, began earlier, and sometimes exceeded the secondary rise which followed it, although the peak rise was less than when injections were made into a systemic vein. Following intraportal injection the rise of arterial pressure was also regularly less than that following intravenous injection and when a secondary rise of portal pressure was distinguishable it followed, by a few seconds, the rise of arterial pressure. In those experiments in which intravenous injection caused the portal pressure initially to fall, such a fall became replaced by an initial rise when injections were made intraportally.

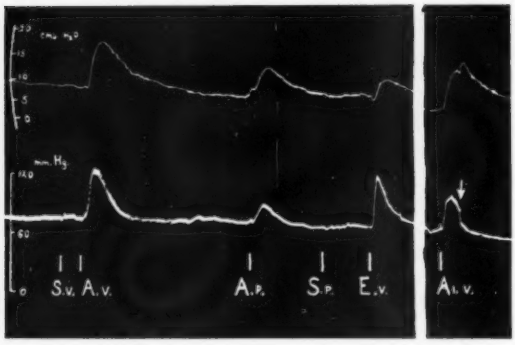


**Fig. 4.** Pressures in the carotid artery and portal vein of a rabbit weighing 1·6 kg. Injections were given as follows: 3·0 ml. of saline intravenously at S.v. and intraportally at S.p.; 0·1 mg. of hypertensin intravenously at A.v. and intraportally at A.p.; and 2·0μg. of adrenaline at E.v. Between the two panels the superior mesenteric artery was occluded. In the second panel 0·07 mg. of hypertensin was injected intravenously and the superior mesenteric artery was released at the arrow.

Some of these facts are illustrated by the first panel of Fig. 4, which shows the pressure in the portal vein and carotid artery of a rabbit weighing 1·6 kg. Intravenous injection of 0·1 mg. of hypertensin at A.v., caused an initial fall of portal pressure followed by a rise which began several seconds after the rise of arterial pressure, and reached its peak after the latter had already begun to decline. A control injection of saline was given at S.v. The same dose of hypertensin was given intraportally at A.p. and a corresponding injection of saline at S.p. It can be seen that the slight fall which occurred previously has been replaced by an initial rise in portal pressure and that the increment in both portal and systemic arterial pressure has been reduced. For comparison, the effect of 2μg. of adrenaline given intravenously is shown at E.v. The initial fall in portal pressure was replaced by an initial rise when (not shown) adrenaline was given intraportally.

*Effect of Injections into the Hepatic Artery.*

Injections made into the hepatic artery through the cut stump of the splenic artery in three cats, caused both the arterial blood pressure and the portal venous pressure to rise, but to a lesser degree than when injections were made intra-

venously. Also, as was the case with the intraportal injections, the portal pressure began to rise several seconds before the rise of arterial pressure.

*Portal Pressures in the Eviscerated Animal.*

The portal pressure in the eviscerated animal was of the order of 3 to 4 cm. of water. No significant change occurred in the portal pressure following the intravenous injection of renin or hypertensin, although the usual change in arterial blood pressure took place. When the animal was eviscerated in the usual way but the hepatic artery was left intact, on the intravenous injection of hypertensin the portal pressure rose from an initial pressure of 5 to 6 cm. by a further 1 to 2 cm. This rise did not take place when the artery was occluded by a clip.

*Effect of Occlusion of the Mesenteric or Hepatic Artery.*

In the cat, when the hepatic artery or the main trunk of the coeliac artery was occluded, an injection of hypertensin which previously had caused a rise of portal pressure had now only a trivial effect. On the other hand, occlusion of the superior mesenteric artery had little effect on the responses of the portal pressure to hypertensin injected intravenously. In the rabbit, a rise of portal pressure occurred as a result of intravenous injection of hypertensin when either the hepatic or the superior mesenteric was occluded, but when both were closed the effect was trivial. In the second panel of Fig. 4, the same rabbit was given an intravenous injection of 0·07 mg. of hypertensin. Between the two panels, the superior mesenteric artery was occluded, and at the arrow, the vessel was released.

*The Venous Outflow from the Liver.*

These experiments were not very satisfactory because the blood pressure, probably as a result of the considerable operative interference, fell to low levels—30 mm. Hg. in one cat and 35 mm. Hg. in another. Also, these preparations were rather insensitive to renin. In the first experiment the outflow increased from 13 to 40 ml. per minute when the blood pressure rose from 35 to 58 mm. Hg. following the injection of renin. At no stage did the blood-flow decrease. In the other experiment, an initial depressor effect was associated with a decrease in outflow and a rise of 15 mm. Hg. above the initial level was associated with a trivial decrease in outflow.

## DISCUSSION.

A possibility which must be considered is that the rise of portal or pulmonary arterial pressure is attributable to impurities. That impurities may contribute to the results is indicated by the fact that samples of renin of equal pressor activity produced unequal degrees of portal or pulmonary hypertension. Some samples of renin produced in the cat two peaks in the pressure in the pulmonary

artery—an initial one possibly attributable to an impurity and a second one synchronous with the peak systemic pressure. Nevertheless, it is unlikely that our results are entirely attributable to impurities, because they have been found with preparations made by so many different methods. For example, the method for making hypertensin differs very much in chemical manipulation from either of the methods used for making renin. Also, for comparison purposes we have been able to use a relatively "pure" sample of hypertensin, obtained through the kindness of Dr. Page, and this gave us similar results.

*The Pressure in the Portal Vein.*

The rise of portal pressure following the intravenous injection of renin or hypertensin may be attributable to one or more of the following factors:

1. A rise of systemic venous pressure.
2. Obstruction of the portal vein or venules by vascular constriction or by pressure from neighbouring tissue.
3. Constriction of the hepatic veins or venules.
4. Transmission of pressure or increased inflow from the arterial side, through the hepatic or mesenteric vessels.

The action of hypertensin has been explored in the cat and to a lesser extent in the rabbit. It is clear that the rise of portal pressure is not due to the first of the abovementioned factors because it is not commensurate with changes in the general venous pressure which in any case were usually negligible. Moreover, it does not occur in the eviscerated preparation.

We found, so far as the cat is concerned, whether "intact" or eviscerated, that a rise of portal pressure depended on the patency of the hepatic artery. Since this vessel supplies the walls of intrahepatic branches of the portal vein and its blood is eventually discharged by the hepatic veins, it might be thought that its patency simply permitted the active substances to be carried to the liver, there to act on either (or both) the portal or hepatic venules. However, the main rise of portal pressure cannot be attributable to constriction of either hepatic or portal vessels because of the results of intraportal or intra-arterial injections, neither of which caused, for a given dosage, as big a rise of portal pressure as did simple intravenous injection. Had the rise of portal pressure been mainly attributable to portal constriction, it should have been greatly increased when hypertensin was given by a more direct vascular route to the liver.

Therefore, we are left with the possibility that the rise of portal pressure is due mainly to an increased pressure in the ultimate ramifications of the arterial supply. In the cat the main field where this occurs is in the hepatic rather than the mesenteric distribution, whereas in the rabbit both fields are important. In these circumstances the pressure in the portal vein may rise because of (a) a transference of pressure through actively or passively dilated arterioles and

capillaries, or (b) a direct compression of portal venules because of a rise of pressure in the surrounding tissue. That the latter is not the important factor is indicated by the fact that the rise of portal pressure takes place when, as in the eviscerated animal, the hepatic artery represents the entire blood supply to the liver. There is, however, no evidence that hypertensin actively dilates the hepatic vessels because of the relatively insignificant rise of portal pressure which occurred when it was given intra-arterially. Indeed, it would be surprising if an active dilatation did in fact occur, since hypertensin constricts every other vascular field where its action has been investigated. Recently Abell and Page (1946) have shown by direct observation, that it constricts the mesenteric arteries and veins of the rabbit.

Therefore, we are faced with the position that the action of hypertensin on the hepatic vessels is weaker or more transient than on some other vascular fields. This would explain why the main rise of portal pressure begins, usually, several seconds after the rise of arterial pressure commences, and may continue to rise after the latter has passed its peak.

One possibility which must be kept in mind is that as a result of opening the peritoneal cavity, the splanchnic vessels are "shocked" and respond passively. Such a criticism might also be levelled against the similar observations to these which were made by McMichael (1932) on the action of adrenaline. Such a possibility may, for example, account for the greater significance of the patency of the superior mesenteric artery in the rabbit, in which it was necessary in setting up the preparation, to handle the intestines more than in the cat. Nevertheless, it is unlikely that the whole phenomenon is attributable to this cause; the vessels have by no means become inert tubes. For example, in the cat and dog the rise of portal pressure is, for a given rise of blood pressure, less than that due to adrenaline, whereas in the rabbit the very reverse is the case. Moreover, in all three species, pituitrin causes a sharp fall in portal pressure, and sometimes with hypertensin itself, a fall precedes the rise.

The discussion so far has taken no account of the initial stages of the rise of portal pressure. This rise usually began at the same time as the rise of arterial pressure, except when injections were made, by the hepatic artery or portal vein, in which case the rise of portal pressure preceded it. In some experiments a two-phase effect could be observed in the portal pressure, a secondary or main rise beginning several seconds after the beginning of the rise in arterial pressure. It was the initial rise which became exaggerated and commenced earlier when intra-portal or intrahepatic injections were made. These observations indicate that the initial stage of the rise of portal pressure is due, as in the case with adrenaline (McMichael, 1932), to a direct effect of hypertensin in the liver. This effect may be due to portal or hepatic vein constriction. Plethysmograph studies which may have helped to distinguish these were not made. The transient fall of portal pressure which sometimes preceded the main rise was (except when associated with

an initial depressor effect) probably attributable to a decrease in liver inflow, such as regularly occurs with pituitrin. It was never observed following intraportal injections.

Another question is to what extent portal or hepatic obstruction may contribute to the main rise of portal pressure. It is already clear from the above that the most significant factor is a transference of pressure from the hepatic artery. One of the outflow experiments indicated that no hepatic vascular obstruction was present, for the outflow increased out of proportion to the rise in either hepatic arterial or portal venous pressure. The other experiment, which showed no significant change in outflow during the systemic hypertension, indicated that there had been an increase in the resistance in the liver.

*The Pressure in the Pulmonary Artery.*

This calls for little discussion. So far as the dog is concerned, the results are consistent with the view that in this species, renin is responsible for renal hypertension which is not associated with a rise of pressure in the pulmonary artery (Katz *et al.*, 1939). So far as we know there are no records of pulmonary arterial pressures in experimental hypertension in cat or rabbit. The mechanism of the rise of pulmonary arterial pressure following the injection of renin or hypertensin has not been explored beyond the fact that it is not due to "back-pressure." From the observations of others (Hill and Andrus, 1941; Lorber, 1941; Bradley and Parker, 1941; Taylor and Page, 1943; and Middleton and Wiggers, 1944) on the action of renin or hypertensin on the cardiac output, it is, however, unlikely that the rise is cardiac in origin.

## SUMMARY.

In the cat and rabbit renin or hypertensin causes a small but significant rise in the pressure in the pulmonary artery. This is not attributable to "back-pressure."

Renin or hypertensin causes, when injected intravenously, a rise of pressure in the portal vein. When injections are made into either the portal vein or hepatic artery, the rise of both the systemic arterial and portal venous pressures is less than when injections are made intravenously. The rise of portal pressure is not due to a general rise of venous pressure and only in a minor degree to constriction of hepatic or portal veins. It is mainly due to transmission of pressure from the hepatic artery, constriction of the portal vein being responsible for the initial part of the rise. Thus, the effect of hypertensin in this respect, resembles the action of adrenaline.

In the cat the effect of hypertensin on the portal venous pressure was, for a given rise of systemic arterial pressure, less than that due to adrenaline, whereas in the rabbit the reverse was the case.

In the dog there was no significant change in the pressure in the pulmonary artery when renin or hypertensin was injected.

Renin injections caused a rise of pressure in the portal vein; hypertensin had only a trivial effect.

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# THE NATURE OF MUMPS VIRUS ACTION ON RED CELLS

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In 1942 Enders and Cohen discovered that the parotid glands of monkeys infected with mumps contained an antigen which reacted specifically in the complement fixation test with convalescent sera from man and monkeys. After several unsuccessful attempts by other workers, Habel (1945) found that mumps virus could be cultivated in the chick embryo by inoculation of parotid gland material from an infected monkey into either the yolk sac, amniotic cavity or allantoic cavity of developing eggs. After a suitable period of incubation, the virus content of the embryonic fluids could be estimated quantitatively by using them as antigen in the complement fixation test and titrating against mumps convalescent sera. Conversely the antibody content of sera could be estimated by titration against infected embryonic fluids of known potency. Later in the same year Levens and Enders (1945) found that amniotic and allantoic fluids containing mumps virus would agglutinate fowl erythrocytes and that convalescent sera would specifically inhibit the agglutination.

More recently, Burnet (1946) reported that human red blood cells sensitized by treatment with mumps virus were rendered specifically agglutinable by mumps antibody, and that the reaction could be used as a sensitive quantitative test for antibody in human serum. In regard to this property the behaviour of mumps virus differs from that of the influenza viruses. When human red cells are added to a preparation of any influenza virus, the cells adsorb the virus and agglutinate. The virus subsequently elutes almost completely, leaving relatively unstable cells which can be stabilized by the addition of an appropriate amount of immune serum. Burnet found that human cells treated with amniotic and most allantoic fluid preparations of mumps virus behaved differently. When immune serum was added to the partially stabilized cells they were not stabilized but became more firmly agglutinated. If cells treated with mumps virus were washed and held at 37° C. after treatment they became stable but were agglutinated to high titre by mumps convalescent sera. This phenomenon was only demonstrable at 37° C., as the cells tended to agglutinate spontaneously at lower temperatures.

This paper reports further studies on the cell sensitizing agent (C.S.A.) and the haemagglutinin (HA) and their relationship to one another and to the complement fixing antigen (C.F.A.).

[1] This work was carried out under a grant from the National Health and Medical Research Council, Canberra.

## METHODS.

*Titration of the Complement Fixing Agent (C.F.A.).*

All titrations were carried out according to Bengtson's (1944) method as recommended by Habel (1945). A pool of strongly positive mumps convalescent sera was used in parallel with a pool of negative sera as control. The titre of each mumps antigen was taken as the reciprocal of the highest original dilution of the pooled positive serum which gave a 3+ or 4+ reaction with the undiluted antigen. Appropriate embryonic fluids from normal eggs were titrated in each test as a control on the antigen.

*Titration of the Haemagglutinin (HA).*

This was carried out by the usual method of this laboratory for the titration of influenza virus (Burnet, Beveridge, Bull and Clark, 1942), 2 p.c. suspensions of red cells in normal saline being used. When fowl cells were used the fowl cell-virus mixtures were held at room temperature for 1 hour before reading. When human cells were used the mixtures were held at 37° C. for 1 hour, shaken, and the results read after a further hour at 37° C. The reasons for adopting these methods are discussed in a later section. The titre was expressed as the reciprocal of the virus dilution which gave the standard "+" degree of partial agglutination of the added red cells.

*Titration of Cell Sensitizing Agent (C.S.A.).*

Serial dilutions of mumps allantoic or amniotic fluids 1/1–1/64 were prepared in 1 ml. volumes in calcium saline (0·85 p.c. NaCl, 0·025 p.c. $CaCl_2$). 0·05 ml. of packed washed human red blood cells (group O) was added to each tube. The tubes were incubated for 2 hours at 37° C. after which the cells were resuspended and the tubes centrifuged. The supernatant fluid was removed and the cells in each tube were resuspended in 10 ml. calcium saline giving an 0·5 p.c. suspension of treated or "sensitized" cells. Since "sensitized" cells tend to agglutinate spontaneously at temperatures of less than about 30° C., it was always necessary to restabilize them by holding them at 37° C. for 1–2 hours after resuspension in saline before they could be titrated. It was often found more satisfactory to treat the cells on one day, resuspend them in saline and hold them in the refrigerator overnight, and then allow them to stand at 37° C. for 1–2 hours next morning before titration.

The following method of titration was used. 0·25 ml. of each cell suspension was added to each of two tubes, one containing 0·25 ml. of a 1/200 dilution of a strongly positive mumps convalescent serum STO which had a titre of 1/3,200 against sensitized cells and the other 0·25 ml. calcium saline. The tubes were shaken and the cells allowed to settle at 37° C. for 1½ hours. At the end of that time the tubes containing serum plus cells which had been treated with a dilution of virus containing sufficient C.S.A. to sensitize them, showed a complete hemisphere of agglutinated cells covering the bottom of the tube (++ reaction). Those tubes containing cells which had been treated with a dilution of virus containing insufficient C.S.A. to sensitize them showed only a small button of unagglutinated cells at the bottom of the tube (— reaction). The tube showing a small button or ring of unagglutinated cells surrounded by a hemisphere of agglutinated cells (+ reaction) was taken as the end point. The reciprocal of the virus dilution used to treat the cells in the tube showing this end point was taken as the C.S.A. titre of the virus preparation. The saline-cell mixtures served as control on spontaneous agglutination of the cells. The cells maintained their specific agglutinability until haemolysis began.

It was found that C.S.A. could also be demonstrated using fowl cells instead of human cells but the titre was always lower than that obtained with human cells.

*Strains of Mumps Virus.*

All strains of mumps virus in current use in this laboratory were found to contain C.S.A. Cells which have been sensitized by adsorption and elution of one strain of mumps virus are no longer agglutinable by another strain of mumps virus. Because of the rather irregular growth of most Australian strains in the allantoic cavity, the work described in this paper was carried out with END, an American strain obtained by courtesy of Dr. J. F. Enders.

## EXPERIMENTAL.

*Mumps Haemagglutinin Titres with Human and Fowl Cells.*

In the course of experiments on the HA of mumps virus in which both human and fowl cells were used, it was found that while the HA titre estimated with fowl cells was only slightly affected by the temperature at which the cells were allowed to settle, the titre obtained with human cells at 37° C. was much greater than that obtained at 4° C. This difference was increased if the human cell virus mixtures were shaken and held for a further hour at 37° C. Table 1 shows the results of a typical titration in which several series of the same dilutions of a virus preparation were titrated with fowl and human cells at different temperatures. At the end of one hour the tubes were shaken and the cells allowed to settle for a further hour. The titres obtained when serial dilutions of the virus preparation were held successively at different temperatures is shown in Table 2. It is clear from these results that the increase in titre at 37° C. is irreversible under these conditions, and not merely a temperature effect.

TABLE 1.

*The effect of temperature on the HA.*

| Temperature. | | HA titre. | |
|---|---|---|---|
| | | Fowl. | Human. |
| 4° C. | 1 hour | 120 | 10 |
| | 2 hours | 130 | 10 |
| 20° C. | 1 hour | 150 | 40 |
| | 2 hours | 150 | 40 |
| 37° C. | 1 hour | 160 | 80 |
| | 2 hours | 230 | 160 |

TABLE 2.

*The effect of temperature on progressive readings of the HA titre.*

| Temperature. | Titre. | |
|---|---|---|
| | Fowl. | Human. |
| 4° C. | 120 | 10 |
| 35° C. | 120 | 60 |
| 35° C. | 160 | 160 |
| 4° C. | 160 | 160 |

This increase in titre is only observed when the cell-virus mixtures are held at 37° C. Virus alone failed to show an increase in titre if held at 37° C. for several hours before titration. A similar effect has been described by Anderson (1947) in the case of the virus of Newcastle disease of fowls.

The question arose as to whether this effect was due to a diminished capacity of the cells to adsorb virus at low temperatures, or to the fact that all virus was adsorbed at 4° C. but was incapable of exercising its full agglutinative effect until it had been held in contact with red cells at 37° C.

In order to investigate this point the following experiment was carried out. A sample of mumps virus was diluted out in triplicate and an equal amount of a 2 p.c. human cell suspension was added to each tube. The cells were allowed to settle at 4° C. The results are summarized in Table 3. The titres shown in column I were obtained when the virus-cell mixtures were shaken and the cells allowed to settle at the temperature shown. Those in columns II and III were obtained with cells which, after the first reading at 4° C., were spun down, washed, resuspended to their original volume in saline, and allowed to settle at the temperature shown.

TABLE 3.

*The increase in HA titre after adsorption on to red cells.*

| I. | | II. | | III. | |
|---|---|---|---|---|---|
| Temperature. | Titre. | Temperature. | Titre. | Temperature. | Titre. |
| 4° C. | 20 | 4° C. | 20 | 4° C. | 20 |
| | | Cells washed | | Cells washed | |
| 4° C. | 20 | 4° C. | 30 | 37° C. | 80 |
| 37° C. | 80 | 37° C. | 120 | 37° C. | 140 |

It can be seen that the virus is adsorbed at 4° C. but exposure of the virus-cell mixtures to 37° C. is necessary for the full agglutination titre to be reached. This suggests that mumps virus, like Newcastle disease virus (Anderson, 1947), is present in the embryonic fluids in loosely aggregated form and that adsorption

to human red cells in the cold takes place mainly in that form. At 37° C. disaggregation is probably accelerated by increased Brownian movement, particularly when associated with mechanical shaking at intervals. If two component virus particles of an aggregate are specifically adsorbed to receptors on two different red cells, any relative movement of the cells will break up the aggregate into two parts. This process would rapidly lead to a state in which each virus particle could exert its full agglutinative power.

*The Relationship Between the HA and C.S.A. Titres of Mumps Virus.*

The titres of a number of different preparations of mumps allantoic fluid are set down in Table 4.

TABLE 4.

*Relationship between HA and C.S.A. titres of nineteen allantoic fluid preparations of mumps virus.*

| Strain. | Haemagglutinin. Human. | Haemagglutinin. Fowl. | C.S.A. Human. | C.S.A. Fowl. |
|---|---|---|---|---|
| END | 160 | 135 | 16 | |
| | 40 | 40 | 4 | |
| | 160 | 110 | 16 | |
| | 160 | 160 | 16 | 8 |
| | 60 | 80 | 8 | 5 |
| | 240 | 240 | 32 | 16 |
| | 80 | 120 | 16 | 8 |
| | 160 | 110 | 16 | 6 |
| | 40 | 80 | 2 | |
| | 160 | 160 | 16 | |
| | 80 | 100 | 8 | |
| | 80 | 75 | 8 | |
| | 240 | 120 | 48 | |
| | 210 | 120 | 24 | |
| SOM | 120 | 240 | 32 | |
| | <10 | 80 | <2 | |
| WAL | 30 | 220 | 16 | |
| | 10 | 120 | 1 | |

As a general rule with strain END the C.S.A. titre determined with human cells was found to be one-tenth of human cell haemagglutinin titre. The C.S.A. titre determined with fowl cells was usually about half that obtained with human cells.

*Separation of the Cell Sensitizing Agent from the Bulk of the Haemagglutinin.*

It is possible to show that C.S.A. can be adsorbed from mumps virus preparations leaving the greater part of the HA intact.

Serial dilutions 1/1–1/64 of high titre mumps allantoic fluid were prepared in 1 ml. volumes in calcium saline. 0·05 ml. of a 20 p.c. suspension of human cells was added to each

tube. The tubes were held at 37° C. for 2 hours. They were then spun, the supernatant fluids were removed to fresh tubes and the cells resuspended in 2 ml. c-lcium saline to give a 0·5 p.c. suspension. 0·05 ml. of 20 p.c. untreated cells was then added to each supernatant fluid and the same process repeated. In all, the virus dilutions were treated with four successive lots of cells. The suspensions of treated cells were stored in the refrigerator until all cell suspensions were available. They were then held at 37° C. for 1–2 hours to ensure stabilization after which each suspension was tested with immune serum diluted 1/200 and saline as a control on stability. After the fourth adsorption the final series of supernatant fluids were all titrated against human and fowl cells to determine their HA activity.

Duplicate series of dilutions of the original virus were held at 37° C. for the duration of the adsorption process and were then titrated for C.S.A. and HA content to determine the degree of natural deterioration of these two activities at 37° C. during this period.

The results of the experiment are shown in Table 5. It will be seen that detectable C.S.A. is removed from successively lower dilutions with each adsorption while the apparent HA is only slightly reduced.

TABLE 5.

*Adsorption of C.S.A. from mumps virus leaving HA.*

C.S.A. titrations.

| Cells treated with. | Cells tested with. | Initial dilutions original virus. 1/1 | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 | C.S.A. titre. |
|---|---|---|---|---|---|---|---|---|---|
| Original virus | STO/200 | ++ | ++ | ++ | ++ | ++ | + | ± | 32 |
| | Saline | — | — | — | — | — | — | — | |
| 1st supernatant | STO/200 | ++ | ++ | ++ | + | ± | — | — | 8 |
| | Saline | — | — | — | — | — | — | — | |
| 2nd supernatant | STO/200 | ++ | ++ | + | ± | — | — | — | 4 |
| | Saline | — | — | — | — | — | — | — | |
| 3rd supernatant | STO/200 | ++ | + | — | — | — | — | — | 2 |
| | Saline | — | — | — | — | — | — | — | |
| Original virus held at 37° C. over period of test | STO/200 | ++ | ++ | ++ | ++ | + | — | — | 16 |
| | Saline | — | — | — | — | — | — | — | |

*HA titres of adsorbed and unadsorbed virus dilutions expressed in terms of undiluted virus.*

| | Cells. | Initial dilutions of original virus. 1/1 | 1/2 | 1/4 | 1/8 | 1/16 | 1/32 | 1/64 |
|---|---|---|---|---|---|---|---|---|
| Original virus | Fowl | 100 | | | | | | |
| | Human | 70 | | | | | | |
| 4th supernatant | Fowl | 75 | 70 | 70 | 50 | 50 | 30 | <64 |
| | Human | 42 | 32 | 36 | 24 | 32 | <32 | |
| Original virus held at 37° C. over period of test | Fowl | 100 | 96 | 96 | 96 | 96 | 96 | 96 |
| | Human | 50 | 42 | 32 | 48 | 32 | 32 | |

*The Effect of Heat on the HA, C.S.A. and C.F.A. of Mumps Virus.*

To observe the effect of heat on the various activities of the virus, samples of high titre mumps allantoic fluid were heated at 40° C., 45° C., 50° C. and 55° C. (all±0·5° C.) for 30 minutes. The HA, C.S.A. and C.F.A. titres of the heated samples were then determined. The results of a typical experiment are shown in Table 6.

TABLE 6.

*The effect of heat on the C.S.A. and HA titres of mumps virus.*

| Temperature to which virus exposed. | C.S.A. | | HA | | Infectivity.* |
|---|---|---|---|---|---|
| | Human. | Fowl. | Human. | Fowl. | |
| Unheated | 16 | 8 | 160 | 145 | 13/13 |
| 40° C. | 16 | 8 | 120 | 160 | 9/12 |
| 45° C. | 8 | 6 | 80 | 145 | 5/13 |
| 50° C. | <2 | <2 | 20 | 105 | 1/12 |
| 55° C. | <2 | <2 | <10 | <10 | 0/13 |

* The denominator denotes the number of eggs alive after 4 days' incubation at 34·5° C. after inoculation, the numerator the number of these eggs in which the virus had multiplied.

The C.S.A. titre estimated with both human and fowl cells was slightly reduced by heating at 45° C. and destroyed by heating at 50° C. The HA titre with both fowl and human cells was only slightly reduced by heating at 45° C. but a more marked reduction was found after heating at 50° C.—the human cell titre showing a more pronounced effect than the fowl cell titre. No HA could be demonstrated after heating at 55° C.

The C.F.A. titre was not affected by heating at any of these temperatures nor by exposure to 65° C. for 30 minutes but was destroyed by boiling for 15 minutes. Infectivity tests on the heated virus samples gave results also shown in Table 6, indicating that the C.S.A. and HA are destroyed at approximately the same temperature as the infectivity of the virus.

*The Effect of Centrifugation on the HA, C.S.A. and C.F.A. of Mumps Virus.*

A sample of mumps allantoic fluid was spun for 10 minutes at 2,500 r.p.m. to remove gross particles. Nine ml. of the fluid were removed and spun at 15,000 r.p.m. for one hour. After spinning, the top third of the fluid was removed and kept, the middle third discarded and the lower third carefully removed from the deposit and kept. The small white deposit obtained was re-suspended in 3 ml. of saline, i.e. one-third of the original volume. The C.S.A. and HA titres of the three fractions were then estimated. The results are shown in Table 7.

TABLE 7.

*The effect of centrifugation on C.S.A. and HA titres of mumps virus.*

| Fraction. | C.S.A. | HA | |
|---|---|---|---|
| | | Human. | Fowl. |
| Original | 8 | 80 | 80 |
| Top 1/3 | <2 | 15 | 20 |
| Lower 1/3 | 3 | 35 | 40 |
| Deposit suspended in 1/3 original volume | 32 (11*) | 180 (60) | 160 (50) |

* The figures in brackets represent the titres of the resuspended ppt in terms of the original volume of fluid from which it was derived, *i.e.*, 1/3 the apparent titre.

The HA and C.S.A. are deposited at approximately the same speed, the slight apparent difference lying well within the limits of experimental error. Experiments described in an earlier paper (Beveridge and Lind, 1946) indicated that the HA and the C.F.A. in mumps allantoic fluid are deposited at approximately the same speed as the virus particles.

*The Effect of Formalin on the HA and C.S.A. of Mumps Virus.*

In determining the HA and C.S.A. titres of two batches of mumps allantoic fluid vaccine, which had been prepared two months previously by the addition of 0·1 p.c. formalin followed by heating at 37° C. for 12 hours and subsequently stored at 4° C., it was found that the preparations would no longer agglutinate human cells and that C.S.A. was no longer detectable with either human or fowl cells although the HA titre estimated with fowl cells had only diminished by one-third. It was decided to study the effect of formalin on the HA, C.S.A. and C.F.A. of the virus.

It was found that after heating the virus at 37° C. for 6 hours in the presence of 0·1 p.c. formalin the fowl cell haemagglutinin titre remained the same, while the human cell titre had diminished to one-third of the original. The C.S.A. was no longer detectable after heating for 2 hours.

After the addition of 0·1 p.c. formalin and storage at 4° C. for 48 hours the fowl cell titre of another virus preparation remained unaltered while the human cell agglutinin titre had diminished to less than half and the C.S.A. titre to one-third that of the original.

The addition of formalin to the virus fluids rendered them anti-complementary and it was not possible to observe the effect of formalin on the C.F. titre.

*The Effect of storage on the HA and C.S.A. Titres of Mumps Virus.*

Unheated mumps virus fluids held at 4° C. showed little diminution in fowl cell agglutinin titre for at least a week. The human cell agglutinin titre diminished

more rapidly and frequently fell to less than half the original titre in a few days. Some preparations maintained their C.S.A. titre unaltered for a week, but usually the titres fell appreciably within this time. At 35° C. all titres diminished more rapidly, the fowl cell titre, however, being the least affected. As mumps allantoic fluid on storage rapidly became anti-complementary, it was not possible to follow the effect of storage on the complement-fixing antigen.

*The Effect of Normal Allantoic Fluid on the HA and C.S.A.*

The inhibitory effect of normal allantoic fluid on fowl cell agglutination by mumps virus was studied by Beveridge and Lind (1946). When serial dilutions of allantoic fluid from normal embryos 10–15 days old were mixed with five agglutinating doses of virus, the inhibition titre of the allantoic fluid was usually in the range 4–8, but occasionally as high as 40. There was some variation between fluids from different embryos of the same age, but the highest titres were obtained with fluids from the oldest ones.

In studying the effect of allantoic and amniotic fluids from 11- and 13-day embryos on both human and fowl cell agglutination and on agglutination of sensitized cells by immune serum the following additional observations were made. Using the methods described by Beveridge and Lind (1946) in which five agglutinating doses of virus were added to serial dilutions of allantoic fluid before the addition of red cells, allantoic fluids from 11-day eggs were found to have only a very slight inhibitory effect on fowl cell agglutination, the inhibitory titres being usually 1 or 2. The effect on agglutination of human cells was more pronounced, the titres being about 5 or 6. With allantoic fluids from normal 13-day eggs the inhibitory titres for fowl cell agglutination ranged from 3 to 6 and for human cell agglutination from 6 to 14. The presence of precipitated urate in some 13-day fluids did not affect the inhibitory titre.

The inhibitory effect of allantoic fluid on fowl cell agglutination was more noticeable with cells from certain fowls than with others, although all the cells used were equally susceptible to agglutination by mumps virus. Also inhibition of fowl cell agglutination was more pronounced at 4° C. than at room temperature, even though a suitable correction was made when necessary for any difference in the five agglutinating doses required at these temperatures.

Some amniotic fluids were tested in both the 11-day and 13-day groups, but in all cases their inhibitory effect was less than that of allantoic fluid.

A pool of mumps allantoic fluids having a C.S.A. titre of 32 estimated in the usual way was added in 0·8 ml. amounts (four sensitizing doses) to 0·92 ml. of falling dilutions 1/1–1/32 of allantoic and amniotic fluids from normal eggs of different ages, 0·5 ml. of a 20 p.c. suspension of human cells was added to each tube and the mixtures held at 37° C. until the cells were stable. Those cells treated in the presence of low dilutions of normal fluids took approximately twice as long to stabilize as those treated in the presence of high dilutions. When stable, the cells were spun down, resuspended in saline to a concentration of 0·5 p.c. held at 37° C. for 1–2 hours and titrated with a 1/200 dilution of immune serum in the usual way.

Nine-day, 11-day and 13-day allantoic fluids were all found to have low inhibitory titres of 2–3 which corresponded to their inhibitory titres for human cell agglutination. A pool of 11-day amniotic fluids had no inhibitory effect whatever and a pool of 13-day amniotic fluids showed an inhibitory titre of 4.

The addition of 11-day and 13-day allantoic fluids to mixtures of immune serum and cells which had been treated with virus in the usual way had no effect on the agglutination of the cells.

*Agglutinin on Stable Cells (A.S.C.).*

As in the case of Newcastle disease virus (Anderson, 1947), human and fowl cells treated with mumps virus will agglutinate normal untreated cells of either species. Serial dilutions 1/1–1/32 of an 0·5 p.c. suspension of mumps treated cells were prepared and an equal amount of an 0·5 p.c. suspension of normal cells was added to each tube. These mixtures plus controls on both cell suspensions were held at 35° C. for 1½ hours and the results read. The results of a typical titration are shown in Table 8. The fowl and human cells used were both treated with the same virus preparation.

TABLE 8.

*Agglutination of normal cells by mumps treated cells.*

| | Titre against normal cells. | |
|---|---|---|
| | Human. | Fowl. |
| Human cells treated with mumps virus | 12 | 8 |
| Fowl cells treated with mumps virus | 11 | 8 |

*The Effect of the Receptor Destroying Enzyme (R.D.E.).*

The receptor destroying enzyme described by Burnet and Stone (1947) was prepared by filtration of 16-hour, semisolid agar cultures of a suitable strain of *V. cholerae*. The agar was first strained through gauze and the expressed fluid centrifuged then passed through a Seitz filter. The pH of the filtrate was adjusted to 7·5–8·5.

This filtrate was used to treat normal human cells in the proportions, 0·1 ml. filtrate (R.D.E.), 1·5 ml. calcium borate buffer saline[2] to 0·05 ml. of packed cells. The mixture was held at 37° C. for one hour, after which the cells were spun down and washed with normal saline. After treatment the cells were no longer agglutinable by mumps virus or by any virus in the series described by Burnet, McCrea and

[2] Calcium borate buffer saline consisted of $Na_2B_4O_7\ 10H_2O$ 0·0520 gm., $H_3BO_3$ 1·203 gm., NaCl 9·00 gm., $CaCl_2$ 1·00 gm. made up to 1 litre in distilled water. The pH was 7·2. The reasons for using this buffer are discussed by Burnet and Stone (1947).

Stone (1946), nor did they show the C.S.A. and A.S.C. phenomena after treatment with mumps virus for the usual time.

TABLE 9.

*Effect of treatment of cells with R.D.E. on C.S.A. titre.*

| Cells. | Titre of mumps convalescent serum. |
|---|---|
| Untreated cells | <100 |
| Cells treated with mumps virus | 3,200 |
| Cells treated with R.D.E. | <100 |
| Cells treated with mumps virus then treated with R.D.E. | 3,200 |
| Cells treated with R.D.E. then treated with mumps virus | <100 |

Cells which had previously been sensitized with mumps virus, on the other hand, after treatment with R.D.E. were still agglutinable by mumps immune sera and still agglutinated normal human cells. Table 9 shows the results obtained when normal and treated cells were used to titrate a mumps immune serum in dilutions 1/100–1/3,200. The effect on the A.S.C. titre of treating cells with R.D.E. before and after treatment with mumps virus is shown in Table 10. 0·5 p.c. suspensions of cells were used in all titrations.

TABLE 10.

*Effect of treatment of cells with R.D.E. on A.S.C. titre.*

| Cells. | A.S.C. titre. |
|---|---|
| Cells treated with mumps virus | 32 |
| Cells treated with mumps virus then treated with R.D.E. | 24 |
| Cells treated with R.D.E. then treated with mumps virus | <1 |

## DISCUSSION.

In the results presented there is nothing to indicate that the activities of mumps virus which have been described are due to anything other than the virus particles themselves. The haemagglutinin, cell sensitizing agent and complement fixing antigen are deposited in the centrifuge at approximately the same rate, indicating that they are particulate and of approximately the same size as mumps and influenza viruses. Further, the haemagglutinin and cell sensitizing agent are destroyed in parallel by heat and at approximately the temperature of inactivation of the virus. The complement fixing antigen is more heat resistant; as with other viruses, particularly those of the psittacosis-lymphogranuloma group, the particles tend to retain their complement fixing power after exposure to temperatures far above those necessary to destroy their infectivity.

Henle, Henle and Harris (1947) have recently differentiated two complement fixing antigens in mumps virus preparations. One corresponds to that with which we have worked and their interpretation of its nature is in accordance with ours. The second antigen appears to be a smaller derivative of the virus which is found chiefly in tissue extracts and which reacts with an antibody appearing earlier in the course of the disease than that reacting with the standard antigen.

If haemagglutinin and cell sensitizing agent are both activities of the virus particles themselves, their apparent dissociation by repeated treatment with red cells calls for some explanation. It is simplest to assume that conditions are similar to those found by Anderson for Newcastle disease virus, viz., that the cell sensitizing agent is essentially that fraction of virus particles which is capable of absorption but not of subsequent elution. The larger fraction of the virus particle population is composed of units which can be repeatedly adsorbed on red cells, cause destruction or distortion of receptors and be subsequently eluted.

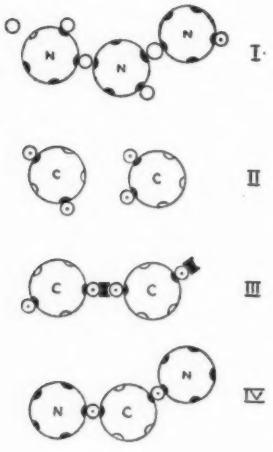


**Fig. 1.** *Diagram to indicate the reaction of mumps virus with human cells.*

The representation is purely schematic; large circles represent red cells, small ones virus particles a central dot indicating that they are inelutable.

Effective receptors are shown as black semicircles, receptors modified by virus action so as to be unavailable to mumps virus by open semicircles on the margin of the cell.

Antibody against mumps virus is shown in III by a biconcave symbol.

I. Agglutination of normal red cells by virus. II. Stability of coated cells alone. III. Agglutination of coated cells by mumps antibody. IV. Agglutination of normal cells by coated cells. N. Normal red cells. C. "Coated" cells.

A suspension of human red cells, coated with mumps virus by Burnet's method, can, therefore, be described as follows: On each cell there are adsorbed several virus particles. Owing to the weakness of their eluting system, these particles are firmly held and protect the corresponding cell receptors from contact either with mumps haemagglutinin or with RDE. Despite the absence of an eluting system, these particles can still unite with intact mumps receptors on normal red cells, so causing agglutination when treated and normal cells are mixed. When immune serum is added to treated cell, antibody forms a bridge between adsorbed particles on different cells and agglutination occurs. A suspension of treated cells is stable because all receptors unoccupied by unelutable particles have been rendered ineffective to hold mumps virus by the activity

of active haemagglutinin. The effect on the receptors is only a partial one (see Burnet, McCrea and Stone, 1946) and by lowering the temperature, sufficient union with damaged receptors on other cells can occur to induce instability of the suspension. The effect of antibody is to allow union between cells *via* the attached unelutable virus particles and hence agglutination.

Evidence of the serological identity of the HA and C.S.A. is provided by the finding that the titre of antihaemagglutinin is proportional to that of sensitized cell agglutinin in immune sera and sera from vaccinated persons (Burnet, 1946).

The strikingly different results obtained in a number of experiments, according to whether fowl or human cells were used to measure the haemagglutinating activity of treated virus, are probably all referable to a firmer adsorption of the virus to fowl cell receptors than to those of human cells. In general, any agent or treatment that tends to inhibit the action of the virus will appear disproportionately effective against human as compared with fowl cells.

At 4° C. union between virus and fowl cell is firm enough to ensure breakage of the forces producing aggregation of virus particles. With human cells at that temperature the virus-cell union usually breaks before the virus-virus union within the aggregate, and only at higher temperatures does the presence of susceptible human cells produce the automatic disaggregation described earlier. Under optimal conditions at 37° C., titres produced with the two types of cell are equivalent. Under the influence of heat, formalin or ageing the diminished adsorptive power of the virus is first shown against human cells, and if nonspecific inhibitors are included in the system they too are apparently more effective when human cells are used as indicators of their effect on the virus. In summary, the virus must be of full normal character to make a satisfactory union with the human cell receptor. Any deviation from the normal greatly weakens or abolishes the union. Union with fowl cell receptors has a higher "tolerance" for minor structural changes in the virus and for the presence of weak inhibitors in the system. This is further borne out by the behaviour of freshly isolated strains of mumps and of strains which have been intensively passaged in eggs, both of which show a variable capacity to agglutinate human cells although the fowl cell agglutination titres may be uniformly high.

It is interesting to note the close resemblance between mumps virus and Newcastle disease virus (Anderson, 1947). Both viruses have the capacity to sensitize red cells, rendering them specifically agglutinable by immune sera and capable of causing agglutination of added normal cells. In both cases there is evidence for aggregation of virus particles in infected fluids.

## SUMMARY.

There is a close relationship between the haemagglutinin, the cell sensitizing agent and the complement fixing antigen in mumps allantoic fluid. These three activities appear to be properties of the virus particles themselves.

Mumps virus agglutinates human as well as fowl cells, but there appears to be a much weaker adsorption of virus particles to human cell receptors than to fowl cell receptors.

There is evidence that mumps virus is present in infected embryonic fluids in an aggregated state.

Cells sensitized with mumps virus have the capacity to agglutinate normal cells.

The mechanisms of haemagglutination, cell sensitization, agglutination of sensitized cells by immune serum and agglutination of normal cells by sensitized cells are discussed.

There is close resemblance between the behaviour of mumps virus and that of Newcastle disease virus.

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